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Pancreatic cancer stem cells and EMT in drug resistance and metastasis

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Abstract

Pancreatic cancer (PC) is an aggressive malignancy with one of the worst outcomes among all cancers. It is the fourth leading cause of cancer death in the United States with a very low five-year survival rate. The high mortality of PC could, in part, be due to their drug resistance characteristics and high propensity for metastasis. Recently, cancer stem cells (CSCs) and epithelial-mesenchymal transition (EMT)-type cells, which shares molecular characteristics with CSCs, have been believed to play critical roles in drug resistance and cancer metastasis as demonstrated in several human malignancies including PC. Thus, the discovery of molecular knowledge of drug resistance and metastasis in relation to CSCs and EMT in PC is becoming an important area of research, and such knowledge is likely to be helpful in the discovery of newer drugs as well as designing novel therapeutic strategies for the treatment of PC with better outcome. In this brief review, we will summarize the current knowledge regarding the CSCs and EMT in the context of drug resistance and metastasis in PC, the molecular events occurring in CSCs and EMT, and the design of novel therapeutic strategies targeting CSCs and EMT-type cells to increase drug sensitivity and suppression of metastasis toward better treatment outcome of patients diagnosed with PC.

Keywords

Pancreatic cancer; cancer stem cells (CSCs); epithelial-mesenchymal transition (EMT)-type cells; drug resistance; metastasis

Introduction

Pancreatic cancer (PC) is an aggressive malignancy with one of the worst outcomes among all cancers. It is the fourth leading cause of cancer death in the United States [1]. For all stages combined, the relative rate of 1-year survival is 24% and only 5% of all patients with PC will survive five years after diagnosis [1]. Even for those people diagnosed with local disease, the 5-year survival is as low as 20% [1]. Approximately, 42,470 people are expected to be diagnosed with PC and 35,240 people are expected to die from this disease in the US in 2009 [2]. The incidence rates of PC have been increasing in women by 0.6% per year since 1994 and the death rate for PC in women has been increasing by 0.1% per year since 1984 [1]. The lethal nature of PC stems from its propensity to rapidly disseminate to the lymphatic system and distant organs. The presence of occult or clinical metastases at the time of diagnosis together with the lack of effective chemotherapies contributes to the high mortality of patients diagnosed with PC, which indeed believed to be due to drug-resistant behavior of PC. The aggressiveness

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of PC could, in part, be due to their intrinsic and extrinsic drug resistance characteristics, and thus the cancer cell resistance to chemotherapeutic agents is a major cause of treatment failure in PC. Therefore, increasing drug sensitivity and inhibiting metastasis are the key steps toward successful treatment of patients diagnosed with this devastating disease.

Recently, cancer stem cells (CSCs) and epithelial-mesenchymal transition (EMT)-type cells have been believed to play critical roles in drug resistance and cancer metastasis. Thus, the molecular knowledge of drug resistance and metastasis with respect to CSCs and EMT in PC is considered very important, and the gain of such knowledge is likely to be helpful not only in the discovery of newer drugs but also in the design of novel therapeutic strategies for the treatment of PC with better outcome. The following sections will summarize what we know regarding CSCs and EMT in the context of drug resistance and metastasis in PC, the molecular events that occurs in CSCs and EMT, and the design of novel therapeutic strategies targeting CSCs and EMT for increasing drug sensitivity and the suppression of metastasis toward better treatment outcome of patients diagnosed with PC.

Pancreatic cancer stem cells (CSCs)

It has been well known that human cancer is composed of a heterogeneous population of cells which have different proliferative potential and they response differentially to chemotherapy and radiotherapy. Among cancer cells, a small population of cancer cells has been identified as cancer stem cells (CSCs) which possess the ability to self-renew and generate the diverse cell population that comprise the cancer mass [3]. A growing body of evidence now supports the concept that cancers are diseases driven by subpopulation of self renewing CSCs, which have been found in hematopoietic [4,5] and solid tumors including brain tumor [6], breast [7], head and neck [8,9], colon [10], lung [11], prostate [12], ovarian [13], and PC [14,15]. The CSCs not only possess the capacity for self-renewal but also have the potential and the ability to drive continued expansion of the population of malignant cells with invasive and metastatic propensity [16]. Moreover, CSCs also show resistance to a number of conventional therapies including chemotherapy and radiotherapy [17,18], which may explain why it is difficult to completely eradicate cancer and why recurrence is an ever-present threat.

To identify and isolate CSC from whole tumor cell population, several cell surface markers including CD24, CD34, CD44, CD133, CD139, CD166, and ESA have been used together with cell sorting [13,19–22] by flow cytometry (FACS analysis). Among them, CD44, CD133, and ESA are more frequently used for the isolation of CSCs from different type of tumors. However, it is important to note that no unique marker for isolation of CSCs from different types of tumors has been found so far, suggesting that combination of several markers could increase the purity of isolated CSCs.

In 2007, two groups of investigators found the presence of CSCs in human PC using two different sets of cell surface markers [14,15]. Li et al firstly described that the $CD44^+/CD24^+/ESA^+$ PC cells, which counted for 0.2–0.8% of PC cells, showed the stem cell properties of self-renewal, the ability to produce differentiated progeny, and increased expression of the developmental signaling molecule sonic hedgehog [14]. They also found that PC cells with the $CD44^+/CD24^+/ESA^+$ phenotype isolated from PC tissue had a 100-fold increased tumorigenic potential compared with marker-negative cancer cells. Furthermore, 50% of animals injected with as few as 100 $CD44^+/CD24^+/ESA^+$ cells formed tumors that were histologically indistinguishable from the original human tumors [14]. Shortly thereafter, Hermann et al reported that human PC tissue contained CSCs defined by CD133 expression [15]. These $CD133^+$ PC cells were exclusively tumorigenic. The results from these two groups clearly demonstrated that there is a small population of PC cells which possess the distinct cell surface markers commonly found in other organ-specific CSCs, and these cells showed properties of self-

renewal, and the increased tumorigenic potential, suggesting that these cells are authentic PC-specific CSCs. Moreover, their data suggest that a combination of the cell surface maker $CD44^+/CD24^+/CD133^+/ESA^+$ could be used to isolate the pancreas-specific CSCs from human PC tissues.

Pancreatic CSCs are drug resistance with propensity to metastasis

Over the past years, emerging evidences have shown that CSCs are largely involved in the drug resistance and metastasis [23–29]. The CSCs also contribute to radioresistance through preferential activation of the DNA damage checkpoint response and an increase in DNA repair capacity [17]. It has been found that the fraction of glioma cells expressing CD133, a marker for brain cancer stem cells, was enriched after radiation in gliomas [17]. The $CD133^+$ glioma cells survived after ionizing radiation showed increased proportions relative to most tumor cells which lack CD133 expression. These results suggest that the $CD133^+$ tumor cells represent glioma CSCs that confers glioma radioresistance. It has been reported that CSCs in mouse mammary tumors may contribute to cisplatin resistance in a mouse model [30]. Similarly, CSCs in colorectal cancers are also believed to be responsible for the resistance to chemotherapeutic drugs [10,31]. Charafe-Jauffret et al have isolated breast cancer cell lines which contain functional CSCs with high metastatic capacity and a distinct molecular signature of CSCs [27], suggesting that breast cancer CSCs are involved in breast cancer metastasis.

It has been known that pancreatic CSCs are implicated in drug resistance and that the drug resistant cells from PC are more tumorigenic and metastatic *in vitro* and *in vivo*. Hermann et al have found that human PC tissues contains pancreatic $CD133^+$ CSCs that are exclusively tumorigenic and highly resistant to standard chemotherapy [15]. They also observed a distinct subpopulation of $CD133^+/CXCR4^+$ CSCs in the invasive front of pancreatic tumors, suggesting the metastatic phenotype of the individual tumor cells. Depletion of the CSC pool for these migrating CSCs virtually abrogated the metastatic phenotype of pancreatic tumors without affecting their tumorigenic potential. These findings demonstrate that pancreatic CSCs contribute to the drug resistance and metastasis. In another study investigating pancreatic CSCs in drug resistance, high-dose gemcitabine treatment was used to eliminate most of the cancer cells [32]. After the treatment, $CD44^+$ cells proliferated and reconstituted the population of resistant cells, suggesting that pancreatic CSCs are gemcitabine resistant. In human PC samples, CD44 expression was correlated with histologic grade, and the patients with CD44-positive tumors showed poor prognosis [32]. These data suggest that PC stem-like cells were expanded during the acquisition of gemcitabine resistance.

EMT in pancreatic cancer

EMT is a process by which epithelial cells undergo remarkable morphological changes characterized by a transition from epithelial cobblestone phenotype to elongated fibroblastic phenotype. EMT was originally identified as a crucial differentiation and morphogenetic physiological process during embryogenesis [33]. Currently, EMT is recognized as pathological mechanism during the progression of various diseases including inflammation, fibrosis and cancers. Alterations in the expression of critical molecules have been observed during the acquisition of EMT phenotype, consistent with their association in cellular signal transduction pathways. In the processes of EMT, cells lose epithelial cell-cell junction, actin cytoskeleton reorganization, and the expression of proteins that promote cell-cell contact such as E-cadherin, γ-catenin, and zonula occludens-1 (ZO-1), and gain mesenchymal molecular markers such as vimentin, fibronectin, α-smooth muscle actin (SMA), fibroblast-specific protein-1, and N-cadherin [34]. Under most circumstances, the cells that are undergoing EMT show down-regulation of E-cadherin which is a cell-cell adhesion molecule and a calcium-dependent transmembrane glycoprotein present in the most epithelial cells in adult tissues. Emerging

evidence also suggest that the process of EMT is triggered by complex molecular interplay between extra-cellular signals and growth factors. The extra-cellular signals and growth factors involved in the EMT process include type I and III collagen, matrix metalloproteinases-2 (MMP-2), MMP-3, MMP-9, transforming growth factor-β (TGF-β), fibroblast growth factor (FGF), epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) A and B [35–39]. Moreover, a number of factors that transcriptionally repress E-cadherin and regulate other EMT markers have emerged as potent EMT drivers during normal development and cancer progression. The transcription factors involved in the regulation of EMT markers include the zinc finger binding transcription factors Snail homologues (Snail1, Snail2/Slug, and Snail3) and several other basic helix-loop-helix transcription factors such as Twist, ZEB1, ZEB2/SIP1, and TCF3/E47/E12 [40,41]. It is now more clear that the down-regulation and relocation of E-cadherin and ZO-1, the translocation of β-catenin from cell membrane to nucleus, the overexpression of vimentin and fibronectin, and the activation of Slug, Twist, and ZEB1 transcription factors, result in the induction of EMT phenotype [40,42–47].

We and other investigators have reported the EMT phenomenon in several PC cell lines and surgically resected pancreatic cancers [48–55]. We found that several PC cell lines including L3.6pl, Colo357, BxPC-3, and HPAC cells showed strong expression of epithelial marker E-cadherin while another group of PC cells (MiaPaCa-2, Panc-1, and Aspc-1 cells) exhibited strong expression of mesenchymal makers including vimentin and ZEB-1 at mRNA and protein levels [48]. Arumugam et al also reported similar observations showing that L3.6pl, BxPC-3, CFPAC-1, and SU86.86 cells had high expression of E-cadherin and low expression of ZEB-1 while low level of E-cadherin and high level of ZEB-1 were observed in PANC-1, Hs766T, AsPC-1, MIAPaCa-2, and MPanc96 cells [49]. These results suggest that a population of distinct PC cells, which show EMT characteristics, exists in pancreatic tumors. These EMT cells in PC could be responsible for the progressive characteristics of PC. In a study investigating the expression of EMT markers such as low E-cadherin, high fibronectin, and vimentin in resected human PC, immunohistochemical staining was performed on formalin-fixed paraffin-embedded tissues using monoclonal antibodies against vimentin, fibronectin, E-cadherin, and p-Erk [53]. It has been found that fibronectin overexpression correlated with the presence of vimentin ($p = 0.0048$) and activated Erk ($p = 0.0264$). There was a borderline association of fibronectin with worsening grade ($p = 0.06$). Importantly, increased fibronectin or vimentin and decreased E-cadherin correlated with poor survival, suggesting that EMT is associated with poor survival in surgically resected pancreatic adenocarcinoma [53]. The expression level of E-cadherin, N-cadherin, vimentin, and TGF-β was also investigated in pancreatic primary and metastatic tumors [55]. N-cadherin expression was observed in 43% (13/30) of primary tumors and in 53% (8/15) of metastatic tumors. N-cadherin expression correlated with neural invasion ($P = 0.008$) and histological type ($P = 0.043$). Vimentin was observed in a few cancer cells of primary tumor but was substantially expressed in liver metastasis. Moreover, it is known that TGF could stimulate N-cadherin and vimentin protein expression and decrease E-cadherin expression in Panc-1 cells with morphological changes [55]. These studies provide the morphological and molecular evidence of EMT in PC cell lines and clinical pancreatic carcinoma tissues.

EMT is implicated in drug resistance and metastasis in PC

Recent studies have shown that EMT is associated with drug resistance and cancer cell metastasis [34,56–60]. Emerging evidence have implicated EMT with the conversion of early stage tumors into invasive malignancies accompanied by increased cell motility and invasion [61], and these processes are consistent with the acquisition of “cancer stem-like cell” phenotype that is also known as “stemness” [62]. For most epithelial tumors, progression toward malignancy is accompanied by a loss of epithelial differentiation and a shift towards mesenchymal phenotype, leading to enhanced cancer cell migration and invasion [61]. Studies have also shown

that cancer cells in the tumor center remain positive for the expression of E-cadherin and cytoplasmic β-catenin; however, the tumor cells in the periphery with propensity for invasion display loss of surface E-cadherin and up-regulation of vimentin, the typical characteristics of EMT phenotype [63]. Highly invasive breast cancer cells have been studied and selected, and these selected invasive cells displayed EMT characteristics and dramatically enhanced invasive ability with decreased level of E-cadherin and increased vimentin, fibronectin, Twist, and AKT2 [60]. Recent findings also suggest that metastasis may be critically dependent on the ability of cancer cells to acquire EMT characteristics [33,64]. EMT has been shown to be important on conferring drug resistance characteristics to cancer cells against conventional therapeutics including taxol, vincristine, oxaliplatin, or epidermal growth factor receptor (EGFR) targeted therapy [59,65], which is consistent with drug resistance characteristics of CSCs.

We have reported that EMT in pancreatic cancer influences the sensitivity of cancer cells to drug treatment. We found that gemcitabine-sensitive PC cells including L3.6pl, Colo357, BxPC-3, and HPAC cells showed strong expression of epithelial marker E-cadherin while gemcitabine-resistant PC cells, MiaPaCa-2, Panc-1, and Aspc-1 exhibited strong expression of mesenchymal makers including vimentin and ZEB-1 at mRNA and protein levels, suggesting that the gemcitabine-resistant cells possess EMT characteristics [48]. Arumugam et al also reported that L3.6pl, BxPC-3, CFPAC-1, and SU86.86 cells were sensitive while PANC-1, Hs766T, AsPC-1, MIAPaCa-2, and MPanc96 cells which showed EMT characteristics were resistant to three conventional chemotherapeutic agents, gemcitabine, 5-fluorouracil (5-FU), and cisplatin [49], consistent with our findings. EMT is also related to the high metastatic ability of PC cells. To study the role of EMT in PC metastasis, highly metastatic PC cell populations were selected by serial *in vivo* passaging of parental cells with low metastatic potential and characterized by global gene expression profiling, chromatin immunoprecipitation, and *in vivo* metastatic assay [66]. The results showed that *in vivo* selection of highly metastatic PC cells induced EMT with the loss of E-cadherin expression and the up-regulation of mesenchymal marker Snail. Inactivation of E-cadherin in parental cells also induced EMT and increased metastasis *in vivo*. Silencing of E-cadherin in highly metastatic cells was mediated by a transcriptional repressor complex containing Snail, HDAC1, and HDAC2. These results suggest that EMT is responsible for the high propensity of metastasis in PC.

Molecular alterations in the formation of CSCs and EMT

Because CSCs and EMT have been believed to be responsible for drug resistance and cancer metastasis, it is important to uncover the molecular mechanism(s) involved in the formation of CSCs and EMT so that novel therapeutic strategies could be designed for the inhibition of cancer progression and metastasis. The formation of CSCs and EMT is a dynamic process and it is triggered by the interplay of multiple cellular signaling pathways such as Hedgehog, Notch, PDGF, Wnt, TGF-β, Akt, and NF-κB signaling pathways [50,51,67–71]. Emerging evidence also implicated the critical role of several microRNAs (miRNAs) in the processes of EMT [72,73]. In addition, experimental evidence has shown that the presence of hypoxia can also induce EMT [43,74].

Deregulation of Hedgehog signaling has been found in the formation of CSCs and EMT. Hedgehog (Hh) signaling is an essential pathway for embryonic pancreatic development. It has been known that Hh signaling plays important roles in proper tissue morphogenesis and organ formation during the developing gastrointestinal tract. Hedgehog ligands including sonic hedgehog (Shh) are expressed throughout the endodermal epithelium at early embryonic stages but excluded from the region that forms the pancreas. Deregulation of the Hh pathway has been implicated in a variety of cancers including PC. It has been reported that overexpression of Shh may contribute to pancreatic tumorigenesis. Thayer et al found that no Shh was detected

in the islets, acini, or ductal epithelium of normal pancreas while Shh was aberrantly expressed in 70% of specimens from the patients with pancreatic adenocarcinoma, suggesting that Shh is a mediator of pancreatic cancer tumorigenesis [75]. The down-regulation of Shh by cyclopamine, an inhibitor of Shh, can reduce the growth and viability of PC cells, suggesting that targeting Shh signaling may be an effective novel approach for the treatment of PC [76]. Importantly, pancreatic CSCs demonstrate up-regulation of sonic hedgehog [14], and the down-regulation of hedgehog signaling by the inhibitors of hedgehog resulted in the inhibition of CSCs and EMT with down-regulation of Snail and up-regulation of E-cadherin, leading to the inhibition of invasion and metastasis in PC [77,78].

In addition to the hedgehog signaling, another important developmental signaling pathways such as Notch signaling is involved in cell proliferation, survival, apoptosis, and differentiation which affects the development and function of many organs [79]. To date, in mammals, the Notch family of trans-membrane receptors consists of four members: Notch-1-4. Mammals also express Notch ligands including Dll-1 (Delta-like 1), Dll-3 (Delta-like 3), Dll-4 (Delta-like 4), Jagged-1 and Jagged-2 [79]. Notch signaling is initiated when Notch ligand binds to an adjacent Notch receptor between two neighboring cells. Upon activation, Notch is cleaved, releasing the intracellular domain of the Notch (ICN) through a cascade of proteolytic cleavages. Recently, Notch signal pathway has been found to be a key regulator in the induction of EMT [70,71,80]. Notch activation in endothelial cells results in morphological, phenotypic, and functional changes consistent with mesenchymal transformation. These changes include down-regulation of endothelial markers (VE-cadherin, Tie1, Tie2, platelet-endothelial cell adhesion molecule-1, and endothelial NO synthase) and up-regulation of mesenchymal markers (α-SMA, fibronectin, and platelet-derived growth factor receptors) [81]. Therefore, it is believed that Notch-induced EMT is restricted to cells expressing activated Notch. Moreover, Jagged-1 stimulation of endothelial cells is known to induce a similar mesenchymal transformation, suggesting that Jagged-1 mediated activation of Notch signaling is important during the induction of EMT [81]. Notch also interacts with several transcription and growth factors including Snail, Slug, and TGF-β in the processes of EMT. Notch has been shown to promote EMT through the regulation of Snail. For example, over-expression of Notch-1 in immortalized endothelial cells *in vitro* could induce Snail [70], which could bind to the E-boxes of human E-cadherin promoter and function as a repressor of E-cadherin gene expression [82]. Thus, Notch induced Snail over-expression is likely to down-regulate E-cadherin expression, resulting in the acquisition of EMT. In addition, Notch could induce EMT through stabilizing Snail-1 protein under hypoxic condition [80]. It has been reported that Slug is a direct target of Notch and that the Notch signaling directly stimulates Slug promoter, resulting in the up-regulation of Slug and the formation of EMT [83]. In addition, Slug was found to be essential for Notch-mediated repression of E-cadherin, resulting in β-catenin activation and EMT [84]. It has been found that TGF-β can induce the expression of Notch ligands [85] and that TGF-β-induced EMT could be blocked by Hey-1 or Jagged-1 RNA silencing or by chemical inactivation of Notch [71]. We have also found that Notch-2 and its ligand, Jagged-1, are highly up-regulated in gemcitabine resistant PC cells, which show the acquisition of EMT phenotype [51]. These findings clearly suggest the important roles of Notch signaling in the formation of EMT.

Notch ligand, Jagged-1 has been found to be an evolutionarily conserved target of Wnt/β-catenin signaling pathway in progenitor cells, suggesting the participation of Wnt signaling in CSCs and EMT [86]. Indeed, Wnt signaling has been shown to play important roles in both self renewal and carcinogenesis in a variety of cancer. Jagged-1 expression on progenitor cells induces self-renewal of stem cells due to canonical Wnt signaling activation and Notch signaling activation [86], demonstrating that Wnt and Notch signaling are important in maintaining the homeostasis of stem and progenitor cells. Moreover, inhibition of Wnt signaling resulted in the re-expression of breast epithelial differentiation markers and repression of EMT transcription factors Slug and Twist [57]. In colorectal cancer cell lines,

overexpression of Snail leads to increased expression of Wnt target genes through the interaction with β-catenin whereas down-regulation of endogenous Snail by siRNA reduces target gene expression [42]. Collectively, these results provide a molecular link between self-renewal, EMT, and Wnt signaling.

Moreover, PDGF signaling has been reported to regulate the expression of the Notch-1 and play important roles in cancer cell growth, invasion and metastasis. Importantly, we have found that PDGF signaling contributes to EMT phenotype, resulting in the aggressiveness of tumor cells such as increased invasion and angiogenesis characteristics [87]. We observed that over-expression of PDGF-D resulted in a significant induction of EMT as shown by changes in cellular morphology concomitant with the loss of E-cadherin and ZO-1, and gain of vimentin [87], suggesting the important roles of PDGF signaling in the induction of EMT phenotype.

In recent years, emerging evidence implicated the critical role of microRNAs (miRNAs) in the processes of EMT. The miRNAs elicit their regulatory effects by imperfectly binding to the 3′ untranslated region (3′UTR) of target mRNA, causing either degradation of mRNA or inhibition of their translation to functional proteins [88,89]. The expressions of miRNAs have been recognized as integral components of many normal biological processes, such as those involving cell proliferation, differentiation, apoptosis, and stress resistance [90]. Moreover, it has been recently suggested that aberrant expression of miRNAs is associated with the development and progression cancer. More importantly, miRNAs have been found to be the regulators of EMT through the regulation of E-cadherin and other molecules [72], and thus miRNA appears to play important roles in cancer development and progression [91,92]. We have investigated the expression level of miR-200 and let-7 in pancreatic EMT-type cells. We found that miR-200b, miR-200c, and miR-200a were down-regulated in gemcitabine-resistant cells, which is consistent with the EMT phenotype of gemcitabine-resistant cells. We also found that many members of the tumor suppressor let-7 family were down-regulated in gemcitabine-resistant cells (EMT-type cells) and these findings are consistent with the aggressiveness of gemcitabine-resistant PC cells [48]. In order to investigate the role of miR-200 family in the reversal of EMT phenotype of gemcitabine-resistant cells, we transfected miR-200a, miR-200b, and miR-200c pre-microRNAs into MiaPaCa-2 cells (gemcitabine resistant) for up to 14 days. We found that the re-expression of miR-200 family in the MiaPaCa-2 cells resulted in the up-regulation of epithelial marker E-cadherin and down-regulation of mesenchymal markers including ZEB1 and vimentin both at the mRNA and protein levels. After 14 days of transfection, the morphology of miR-200 transfected MiaPaCa-2 cells was partially changed from elongated fibroblastoid to epithelial cobblestone-like appearance, and the cells appeared to grow in close contact with each other. These results suggest that the loss of miR-200 family is critical for the acquisition of EMT characteristics and that the re-expression of miR-200 could reverse the EMT phenotype of gemcitabine-resistant cells. Moreover, we have also found that re-expression of miR-200b in PDGF-D overexpression cells led to the reversal of EMT phenotype, which was associated with the down-regulation of ZEB1, ZEB2 and Slug expression and these results were consistent with increased gene expressions of epithelial markers [93]. Moreover, transfection of PDGF-D overexpression cells with miR-200b also inhibited cell migration and invasion with concomitant repression of cell adhesion to culture surface and cell detachment [93]. These findings suggest that miRNAs play important roles in the formation of EMT in cancers including PC. The deregulation of other signaling pathways and molecules (i.e. TGF-β, Akt, PTEN, GSK-3β, NF-κB, etc) also plays roles in the development of CSCs and EMT. The *in vitro* and *in vivo* findings reviewed above demonstrate that a complex signaling network regulates the formation of CSCs and EMT.

Targeting CSCs and EMT to increase drug sensitivity and inhibition of metastasis

Because CSCs and EMT-type cells play important roles in drug resistance and metastasis, novel inhibitors of EMT or agents that could either reverse the EMT phenotype or kill CSCs or EMT-type cells would be a novel strategy for the treatment of cancers including PC, which are highly drug resistant. Agents that could either reduce drug resistance or reverse it would likely be useful for increasing therapeutic activity of conventional therapeutics. Therefore, discovery of precise mechanisms that govern the processes of EMT and the maintenance of cancer stem cell reservoir would likely lead to the exploitation of novel strategies for the inhibition of tumor progression and/or the killing of cancer stem cells in order to achieve complete eradication of tumors. Indeed, very recently, Gupta et al. identified a selective agent that kills cancer stem-like cells (EMT-type cells) very efficiently [94]. They found that salinomycin destroyed cancer stem-like cells selectively. When compared to paclitaxel, which is a commonly used drug for the treatment of many malignancies including breast cancer, salinomycin reduced the proportion of cancer stem cells by more than 100-fold and inhibited mammary tumor growth in mice, which was associated with increased epithelial differentiation of tumor cells [94].

Recently, dietary “natural” compounds have been found to regulate EMT in cancers. We have previously found that isoflavone genistein inhibited cell growth and induced apoptosis in various cancers including PC. We have also found that isoflavone genistein treatment significantly inhibited Akt activity, NF-κB DNA-binding activity, and Notch activation in PC *in vitro* and *in vivo* [95,96]. We have also found that 3,3′-diindolylmethane (DIM) inhibited cell proliferation and induced apoptotic cell death in a variety of cancers including PC through the inhibition of Akt and NF-κB signaling [97]. Therefore, we have investigated whether isoflavone and DIM could reverse EMT [48]. Indeed, we found that DIM and isoflavone treatments increased the level of miR-200 family in MiaPaCa-2 cells (EMT-type cells). After the treatment of PC cells with low concentration of DIM (5 μM) and isoflavone (10 μM) for 21 days, we found up-regulation of epithelial marker E-cadherin and down-regulation of mesenchymal markers, ZEB1, vimentin and slug, at different levels. Immunofluorescence staining showed that E-cadherin was re-distributed in the cytoplasm closer to cell membrane after DIM and isoflavone treatments. Importantly, we also observed that the morphology of MiaPaCa-2 cells changed from elongated fibroblastoid to epithelial cobblestone-like appearance, and it seems that the cell-cell contact was increased after the treatments. These results are consistent with the changes in the expression of miRNAs and EMT markers. We also noted that some PC cells treated with isoflavone showed more elongated shape which was commonly seen in genistein treated cells; however, isoflavone showed stronger effect on E-cadherin expression while DIM exerted stronger effect on ZEB1 expression. These results suggest that both DIM and isoflavone could regulate the expression of EMT markers and indeed could be useful for the reversal of EMT phenotype. Interestingly, the combined effects of isoflavone and DIM could be even better, which requires further in-depth investigation. We further assessed the sensitivity of gemcitabine-resistant cells to gemcitabine after being transfected with miR-200b or treated with DIM or isoflavone. We found that the sensitivity to gemcitabine was significantly increased after miR-200 re-expression compared to control cells. The cells transfected with miR-200b showed 20.8% to 38.2% more inhibition compared to control. The pre-treatment of MiaPaCa-2 cells with DIM or isoflavone also increased the sensitivity of MiaPaCa-2 cells to gemcitabine. These results suggest that miR-200 re-expression, and DIM or isoflavone treatment could partially increase the sensitivity of gemcitabine-resistant cells to gemcitabine possibly through miR-200 mediated reversal of EMT status. These results suggest that isoflavone and DIM could be useful for the treatment of PC especially in combination with existing therapeutic agents.

We have also found that Notch-2 and its ligand, Jagged-1, are highly up-regulated in gemcitabine resistant PC cells, which show EMT phenotype as stated earlier [51]. Importantly, we found that down-regulation of Notch signaling by siRNA approach led to partial reversal of the EMT phenotype, resulting in the mesenchymal-epithelial transition (MET), which was associated with decreased expression of vimentin, ZEB1, Slug, Snail, and NF-κB. These results suggest that the inactivation of Notch signaling by novel strategies could be a potential targeted therapeutic approach for overcoming chemoresistance toward the treatment of PC. In addition, novel strategies targeting Hedgehog signaling by using Hh inhibitors could also be used to suppress CSCs and EMT phenotype for the treatment of PC in the near future.

Conclusion and perspectives

The data from *in vivo* human and animal studies and *in vitro* experiments described above clearly suggest the existence of CSCs and EMT-type cells, which confer drug resistance and leads to metastasis in PC. The regulation of multiple signaling pathways including Hedgehog, Notch, Wnt, PDGF, TGF-β, Akt, NF-κB, and miRNA contributes to the sustenance of CSCs and EMT phenotypic cells that shares many molecular characteristics with CSCs. With the discovery of more molecular knowledge of CSCs and EMT-type cells, novel therapeutic strategies could be designed to target CSCs and EMT-type cells to increase drug sensitivity, which will thereby suppress tumor progression and metastasis. However, more *in vivo* animal studies are needed to investigate the novel therapeutic strategies targeting CSCs and EMT. Recently, Jimeno et al reported a direct PC xenograft model which could be useful as a platform for cancer stem cell therapeutic development [98]. In addition, there are still many unresolved issues regarding CSCs and EMT, such as highly impure population of CSCs, varied reported frequencies of CSCs in the same or different types of tumors, relatively poor overlap between the different makers reported for the CSCs in a given tumor type, the stability of CSC phenotype over time, the relationship between CSCs and EMT, and the complexity of the signaling networks that regulate EMT induction. Nevertheless, owing to the clinical importance of CSCs and EMT in PC, targeting CSCs and EMT-type cells would become an attractive and novel strategy for the treatment of patients diagnosed with PC for which better therapy is urgently needed.

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