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# Vitamin D Receptor Signaling and Pancreatic Cancer Cell EMT

**Zhiwei Li**[1], **Junli Guo**[2], **Keping Xie**[3], and **Shaojiang Zheng**[2]

[1]Department of Gastrointestinal Medical Oncology, Harbin Medical University Cancer Hospital, Harbin, The People's Republic of China

[2] Pathology Department of Affiliated Hospital, Hainan Provincial Key Laboratory of Carcinogenesis and Intervention, Hainan Medical College, Haikou, Hainan 571199, P. R. China

[3]Departments of Gastroenterology, Hepatology & Nutrition, The University of Texas MD Anderson Cancer Center, Houston, Texas

## Abstract

Pancreatic ductal adenocarcinoma remains one of the most lethal of human malignancies. Even in patients who undergo resection, long-term survival rates remain extremely low. A major contributor to the aggressiveness of pancreatic ductal adenocarcinoma is epithelial-to-mesenchymal transition (EMT), a physiologic process of morphological and genetic changes in carcinoma cells from an epithelial phenotype to a mesenchymal phenotype, which is the basis of the high metastatic potential of pancreatic cancer cells. EMT is triggered by various tumor microenvironmental factors, including cytokines, growth factors, and chemotherapeutic agents. This review highlights the growing evidence of the effect of EMT on pancreatic cancer progression, focusing on the interaction of EMT with other pathways central to cancer progression, especially vitamin D receptor signaling. Studies of the signaling pathways that lead to the inactivation of EMT programs during these disease processes are providing new insights into the plasticity of cellular phenotypes and possible therapeutic interventions.

### Keywords

pancreatic ductal adenocarcinoma; Vitamin D receptor; epithelial-to-mesenchymal transition

## Introduction

The mortality rate for patients with pancreatic ductal adenocarcinoma (PDAC) is nearly 75% within one year of diagnosis, the 5-year survival rate is less than 6% (1), and the incidence of this disease appears to be increasing (2). The dismal prognosis of pancreatic cancer is attributable to its tendency toward late presentation, early metastasis, and resistance to therapy. Despite improvements in early diagnosis, surgical techniques, and chemotherapy,

*Corresponding author:* Zhiwei Li, Department of Gastrointestinal Medical Oncology, Harbin Medical University Cancer Hospital, Harbin, The People's Republic of China. Phone: 86-0451-86298278; Fax: 86-0451-86298222; ; Email: lzhw0451@163.com; Keping Xie, Department of Gastroenterology, Hepatology & Nutrition, Unit 955, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: 713-794-5073; Fax: 713-745-3654; ; Email: kepxie@mdanderson.org; or Shaojiang Zheng, Department of Pathology, Hainan Medical College Affiliated Hospital, Haikou, People's Republic of China; Phone: +86-898-66723333; ; Email: zhenghoho@aliyun.com.

most pancreatic cancer patients die owing to the physiological effects of PDAC invasion and metastasis to the regional lymph nodes and/or distant organs (3). Unfortunately, little is known about the reasons for the aggressiveness of PDAC. Therefore, a better understanding of the molecular mechanisms underlying PDAC metastasis is required, and novel, effective prevention and therapeutic modalities are urgently needed to save the lives of patients with PDAC.

Epithelial-to-mesenchymal transition (EMT) plays a crucial role in the invasion and metastasis of PDAC. EMT is characterized by the loss of epithelial cell-cell contacts through the inhibition of epithelial cadherin (E-cadherin), zonula occludens-1, occludin, claudin-1, and claudin-7 and through the acquisition of mesenchymal features such as the upregulation of Slug, Snail, zinc finger E-box-binding homeobox 1 (ZEB1), ZEB2, Twist, and vimentin, and the production of matrix proteins, all of which lead to cell migration and invasion (4). Multiple studies have indicated that EMT occurs in the progression of human cancers; however, no direct evidence has been found to prove this concept, because EMT is transient and lacks specific markers. In addition, the molecular mechanisms by which EMT occurs have not been fully elucidated (5, 6).

One pathway that appears to be involved in EMT regulation in PDAC is vitamin D receptor (VDR) signaling. VDR signaling has multiple antitumor effects, including the negative regulation of cell cycle progression by directly binding to the promoter region and activating the expressions of *p21* and *p27* (7-9), which are two critical cell cycle regulators and are also the direct downstream target genes of Krüppel-like factor 4 (KLF4) (10,11). The induction of *p21* and *p27* can suppress cyclins D1, E, and A and cyclin-dependent kinases 2 and 4 (12) and produce an antiproliferative effect, mainly attributable to cell cycle arrest at the G0/G1 phase (13), in many types of cancer (14). The antitumor activities of the vitamin D3 (VD3)-VDR complex have been demonstrated in a preclinical model and in a clinical trial (15). However, we found that in PDAC the VDR signaling pathway also promotes migration and invasion through the upregulation of the fork-head box M1 (FoxM1) protein and its target gene (e.g. Cyclin D1, Skp2, c-Myc, cluster of differentiation (CD)44, c-Met). The vitamin D active metabolite 1,25 dihydroxyvitamin D(3) (1,25D), mediated via the VDR, directly alters patterns of gene expression and can influence whether the outcome is proliferation, differentiation, or apoptosis. We also found that VDR can regulate the expression of EMT markers. These genomic effects may be a result of both the classical mechanism of VDR recruitment of co-activators on DR3-type vitamin D response elements and non-classical interactions with activated β-catenin on other promoters (16). Therefore, we will review the relationship between VDR signaling and EMT in PDAC.

## 1. Vitamin D and VDR

### 1.1 Discovery of Vitamin D and VDR

Vitamin D was first discovered in the 1920s as a nutritionally essential vitamin for its ability to cure rickets with unknown structure. However, the fact that vitamin D works as a steroid was revealed by the elucidation of vitamin D's chemical structure a few years later. There are two main forms of vitamin D in nature: vitamin D2 (ergocalciferol), which is photochemically synthesized in plants, and vitamin D3 (cholecalciferol), which is

synthesized in the skin of animals and humans in response to sunlight. Until the 1960s, either vitamin D2 or vitamin D3 was believed to be responsible for all the known vitamin D activity (17). Subsequently, an as-yet unknown compound was purified and identified as a steroid vitamin D, 25-hydroxyvitamin D3 (25[OH]D3), in 1968 (18). In 1971 (19-21), it was determined that the steroid vitamin D was a precursor of a new steroid hormone, 1,25D. 1,25D results from the hydroxylation of vitamin D3 by 25-hydroxylase and 1α hydroxylase in the liver and kidney, respectively (22). Since the identification of 1,25D as the active metabolite of vitamin D3, the subcellular localization of this active hormone was found in nuclear fraction (23, 24). Later, the complementary DNA for VDR, which specifically binds 1,25D to nuclear components, was cloned from a chicken intestinal lambda gt11 complementary DNA expression library (25), a rat kidney lambda gt11 expression library (26), and the human intestine and T47D cell complementary DNA library (27).

### 1.2 The Structure and Function of VDR

Human VDR has four major functional domains (28): the highly variable N-terminal domain (A/B region), the highly conserved zinc finger-containing DNA-binding domain (C domain), the flexible hinge region (D region), and the ligand-binding domain (E/F region). The short A/B region is the most divergent among the nuclear receptors and contains autonomous activation function-1, which appears to be inactive (29). The A/B domain is known to be involved in VDR transactivation. VDR's cognate DR3-type vitamin D response element binds as a heterodimer with the ubiquitous retinoid X receptor in the promoter region of target genes, leading to the activation or repression of transcription via interaction with transcriptional cofactors and the basal transcriptional machinery (30). The ligand-binding domain has a characteristic secondary domain structure, which is common for all nuclear receptors. The crystal structure of human VDRΔ ligand-binding domain (Δ means that it is lacking the D hinge domain) shows that it has a canonical shape, with 13 α-helices sandwiched in three layers and a three-stranded β sheet (31). The ligand-binding pocket is rather large, with 1,25D occupying only 56% of this volume (31). The ligand-binding domain is multifunctional and facilitates ligand binding, nuclear localization, dimerization, and interaction with coactivator and co-repressor proteins. Upon binding to a ligand such as 1,25D, VDR is stabilized as a result of phosphorylation at serine 51 and serine 208 (32-35). The ligand-binding domain also contains a dimerization interface and a ligand-dependent transcriptional domain, activation function-2. Ligand binding induces a conformational change of the activation function-2 helix that allows the recruitment of coactivators from the p160 family or the vitamin D3 receptor/thyroid hormone receptor family (36, 37).

## 2. The Role of VDR in Cancer

It is now well established that VDR regulates at least 229 genes through binding to at least 2,776 genomic DNA binding sites (38). The genes include those involved in anti-proliferation, pro-differentiation, anti-inflammation, pro-apoptosis, immune regulation, and many other functions in a tissue- and cell-specific manner (39-45).

### 2.1 Antiproliferative Effects of VDR

VDR, a natural receptor for the secosteroid hormone vitamin D, is a ligand-dependent transcription factor. Vitamin D and its analogues play a pivotal role in anti-proliferative activities in several cancer cell lines (46). Miller *et al.* observed that increasing the concentration of VDR in JCA-1 prostate carcinoma cells was positively associated with antiproliferation induced by 1,25D. Conversely, downregulation of VDR expression in ALVA-31 prostate cancer cells attenuated the ability of 1,25D to inhibit cell growth (47). Keith *et al.* (48) argued that the percentage of VDR-positive cells rather than the absolute level of cellular VDR expression in a heterogenous tumor would be the best predictor of growth inhibition induced by vitamin D compounds. Overall, these studies demonstrated that VDR is required for the antiproliferative effect of 1,25D in cancer cells.

### 2.2 The Role of VDR in Cell Cycle Arrest

previous study, vitamin D3 mediated via Sp1 and NF-Y binding sites upregulated $p27^{Kip1}$, a typical cyclin-dependent kinase inhibitor, so as to inhibit cell cycle progression at the G1/S transition, and Sp1 constitutively bound the $p27^{Kip1}$ promoter and functioned as an anchor protein to recruit VDR to stimulate $p27^{Kip1}$ expression after vitamin D3 treatment (49). Thorne *et al.* (50) found that VDR induced cell cycle arrest through targeting cyclin-dependent kinase N1A, which encodes p21(waf1/cip1) in non-malignant RWPE-1 prostate epithelial cells. VDR dynamically induced individual histone modification patterns specific to each phase of the cell cycle at three VDR binding sites (R1, 2, 3) on the cyclin-dependent kinase N1A promoter. VDR binding to the *MCM7* gene induced H3K9ac enrichment associated with rapid mRNA upregulation to generate miR-106b and to consequently regulate p21(waf1/cip1) expression and cell cycle arrest. Moreover, in breast cancer cell lines, estrogens upregulated VDR expression, thus enhancing cellular sensitivity to circulating 1alpha,25-dihydroxyvitamin D3 (1,25[OH]2D3). The 1,25(OH)2D3-VDR complex then induced *p21* expression, effectively limiting cell proliferation in response to estrogens and growth factors and resulting in cell cycle arrest in the G0/G1 phase and a reduction of cells in the S phase (51).

### 2.3 The Role of VDR in Cell Apoptosis

1,25D interacts with VDR to modulate proliferation and apoptosis in a variety of cell types. Zinser *et al.* (52) showed that cancer cells derived from VDR knock-out animals were completely resistant to 1,25D-mediated growth arrest and apoptosis. Danielsson *et al.* (53) found that VDR ligands could induce apoptosis only in certain melanoma cell lines, suggesting that the effects of VDR ligands on the inhibition of the cell cycle and on the induction of apoptosis are mediated by different genes. In that study, immunohistochemical analysis for cell proliferation and apoptosis was done in mice anterior prostates to provide the evidence that links vitamin D status to the modulation of prostate biology. Kovalenko *et al.* (54) found that low dietary vitamin D intake or deletion of VDR in prostate epithelial cells created an environment in the prostate characterized by high proliferation and low apoptotic rates that may be permissive to events that enhance subsequent prostate carcinogenesis. In MCF-7 human breast cancer cells, 1,25D dependently induced growth arrest and apoptosis (55). Flow cytometric analysis indicated that 1,25D and EB1089

induced cell cycle arrest in G0/G1 that was associated with an accumulation of the hypophosphorylated form of the retinoblastoma protein. MCF-7 cells treated with either 1,25D or EB1089 for 48 hours exhibited characteristics of apoptosis, including cytoplasmic condensation, pyknotic nuclei, condensed chromatin, and DNA fragmentation. Cells treated with either agent exhibited upregulation of proteins associated with mammary gland regression (clusterin and cathepsin B) and downregulation of the anti-apoptotic protein bcl-2 (56).

### 2.4 The Role of VDR in Cancer Invasion

Invasion is an essential component of cancer cell metastasis. Recently, studies using the human epidermoid carcinoma cell line A431 clearly demonstrated that VDR reduced tumor progression. The loss of VDR observed upon the silencing of p63 led to the enhanced invasion of A431 cells, suggesting that the p63-mediated regulation of VDR has a role in inhibiting the migration and invasion of A431 cells (57). Also, the upregulation of the zinc-finger transcription factor Snail has been shown to be linked to the acquisition of the migratory/invasive phenotype, which promotes invasion and metastasis by repressing multiple proteins, including E-cadherin and deltaNp63alpha (58). Interestingly, VDR is known to be repressed by Snail, and a negative correlation between Snail and VDR has been reported in a colon cancer cell line. It is thus likely that Snail represses the deltaNp63alpha-VDR-E-cadherin axis to promote the invasiveness of cancer cells (57). VDR has also been shown to inhibit the invasiveness of prostate cancer cells through binding to vitamin D(3). Tokar *et al.* found that vitamin D(3) exerted its anti-invasive effects by upregulating VDR and decreasing matrix metallopeptidase 9 and matrix metallopeptidase 2 activity (59).

## 3. VDR Signaling and PDAC EMT

### 3.1 EMT of Cancer Cells

Epithelial cancers make up the vast majority of cancer types. During the transition from benign adenoma to malignant carcinoma and metastasis, epithelial tumor cells acquire a de-differentiated, migratory, and invasive behavior. This process of EMT goes along with dramatic changes in cellular morphology, the loss and remodeling of cell-cell and cell-matrix adhesions, and the gain of migratory and invasive capabilities. EMT itself is a multistage process, involving a high degree of cellular plasticity and a large number of distinct genetic and epigenetic alterations, as fully differentiated epithelial cells convert into poorly differentiated, migratory, and invasive mesenchymal cells.

Accumulating evidence has revealed that many growth factors and cytokines as well as cellular signaling pathways could trigger EMT (60, 61), and recently, a plethora of genes have been identified that are critical for EMT and metastasis formation. Notably, the EMT process not only induces increased cancer cell motility and invasiveness but also allows cancer cells to avoid apoptosis, anoikis, oncogene addiction, cellular senescence, and general immune defense. EMT seems to play a critical role in the generation and maintenance of cancer stem cells (CSCs), which is highly consistent with the notion that metastatic cells carry the ability to initiate new tumors (62). EMT requires a loss of cell-cell adhesion and apical-basal polarity as well as the acquisition of a fibroblastoid motile

phenotype. Several transcription factors have been found in recent years to induce EMT, with important implications for tumor progression; however, their effects on cell polarity remain unclear (63). Transcriptional and post-transcriptional regulatory mechanisms mediated by several inducers of EMT, in particular the ZEB and Snail factors, downregulate the expression and/or functional organization of core polarity complexes. These recent observations provide new insights into the relationship between alterations in cell polarity components and EMT in cancer, opening new avenues for these components' potential use as therapeutic targets to prevent tumor progression (63).

Some studies have shown that the aberrant activation of EMT in adult epithelia can promote tumor metastasis by repressing cell adhesion molecules, including E-cadherin. Reduced intracellular adhesion may allow tumor cells to disseminate and spread throughout the body. A number of transcription proteins of the Snail superfamily have been implicated in EMT. These proteins have been shown to be overexpressed in advanced gastrointestinal tumors, including esophageal adenocarcinomas, colorectal carcinomas, and gastric and pancreatic cancers, with a concomitant reduction in the expression of E-cadherin. Regulators of EMT may provide novel clinical targets to detect gastrointestinal cancers early, so that cancers previously associated with a poor prognosis such as pancreatic cancer can be diagnosed before they become inoperable. Furthermore, pharmacological therapies designed to inhibit these proteins will aim to prevent local and distant tumor invasion (64).

### 3.2 Regulation of PDAC Cell EMT

PDAC ranks as the fourth most common cause of cancer death and its incidence is increasing worldwide. The lethal nature of pancreatic cancer is attributed to its high rate of metastasis potential to the lymphatic system and distant organs. The lack of effective therapeutic options contributes to the high mortality rates of patients with PDAC. Recent evidence suggests that EMT plays an important role in disease progression and the development of drug resistance in PDAC. Tumor budding is thought to reflect the process of EMT which allows neoplastic epithelial cells to acquire a mesenchymal phenotype and thus increase their capacity for migration and invasion and become resistant to apoptotic signals. The presence and prognostic significance of tumor budding in PDAC were investigated, and high-grade budding was associated with aggressive clinicopathological features of the tumors as well as worse outcomes of the patients (65). The identification of these EMT phenotypic targets may help develop therapeutic strategies directed specifically against them that could have an impact on drug resistance and invasiveness and thus improve the prognosis of PDAC patients (65).

EMT is a biological process that allows well-differentiated, polarized epithelial cells to undergo a conversion to motile, unpolarized mesenchymal cells. EMT plays a crucial role during implantation, embryogenesis, and organ development (Type 1 EMT); is associated with tissue regeneration and organ fibrosis (Type 2 EMT); and is involved in cancer invasion, metastasis, and drug resistance (Type 3 EMT). Since aggressiveness and drug resistance are hallmarks of PDAC, significant effort has been undertaken in recent years to elucidate molecular EMT mechanisms in this malignancy with such a dismal prognosis. This represents a formidable challenge for several reasons: EMT is a dynamic process, with

regard to both spatial and temporal heterogeneity. Moreover, EMT is induced and regulated by a complex network of traditional signaling pathways and new players like microRNAs.

Interestingly, similar molecular characteristics link EMT-type cells to the concept of CSCs (66). Recent insights regarding the role of CSCs and EMT in tumorigenesis have brought further understanding to the field and have highlighted new therapeutic targets. CSCs are a distinct subset of cancer cells with the ability to differentiate into other cell types and self-renew in order to fuel the maintenance of tumor amplification. EMT endows cancer cells with increased migratory and invasive properties and thus facilitates the initiation of metastasis. EMT is regulated by a complex network of factors, including cytokines, growth factors, aberrant signaling pathways, transcription factors, and the tumor microenvironment. There is emerging evidence that the EMT process may give rise to CSCs or other cells with stem cell-like properties (67). The high mortality rate associated with PDAC could, in part, be due to their drug resistance characteristics and high propensity for metastasis. Recently, CSCs and EMT-type cells that share molecular characteristics with CSCs have been shown to play critical roles in drug resistance and cancer metastasis, as demonstrated in several human malignancies, including PDAC (68). Thus, the relationship between drug resistance and metastasis and CSCs and EMT in PDAC is becoming an important area of research, and such knowledge is likely to be helpful in the discovery of newer drugs as well as in designing novel therapeutic PDAC treatment strategies with better outcomes (68).

### 3.3 VDR and EMT Phenotype Markers

VDR mediates the antitumoral action of the active vitamin D metabolite 1,25D, and vitamin D and VDR may help to block EMT. Studies in breast and colon cancer cells found that VDR, β-catenin, and Snail are interrelated (69). When VDR is activated, it will compete with β-catenin to combine with transcription factor 4, thus inhibiting the activity of β-catenin in colon cancer (70). VDR, which activates *CDH1* expression upon ligand binding, is repressed by Snail but induced by ZEB1 (71). As ligand-activated VDR induces epithelial differentiation and the expression of *CDH1*/E-cadherin and other intercellular adhesion genes, VDR repression by Snail1 and Snail2 guarantees the induction of EMT, even in the presence of 1,25D. This effect seems to be specific to the Snail family of transcription factors, since other EMT inducers such as ZEB1, ZEB2, E47, and Twist1 do not inhibit the human VDR gene promoter (72). Some studies indicate that the transcription factors Snail1 and Snail2 are repressors of VDR and thus of 1,25D action in colon cancer cells (73). Data from colon cancer biopsies indicate that Snail1 and Snail2 may be responsible for VDR downregulation during colon cancer progression (74). Snail-mediated EMT is relevant in the progression of pancreatic cancer, and Snail could be a molecular target for a pancreatic cancer intervention (74). Cancer patients with high levels of Snail1 and Snail2 have lower VDR expression and therefore are resistant to therapy with vitamin D compounds (75).

### 3.4 VDR and EMT in PDAC

Over-expression of FoxM1 caused the acquisition of the EMT phenotype via upregulation of mesenchymal cell markers, including ZEB1, ZEB2, Snail 2, and vimentin, in PDAC cells in one study (76). Consistent with this notion, Huang *et al.* found that FoxM1-Caveolin1 promoted EMT in both mouse and human PDAC cells (77) and in another study

demonstrated that FoxM1c can also contribute to EMT by enhancing urokinase receptor gene transcription (78). Meanwhile, KLF4 was studied as a negative regulatory factor of EMT (79), and increased expression of KLF4 led to the downregulation of Slug and Snail, while knockdown of KLF4 did the opposite. Of note, the increased KLF4 expression significantly upregulated VDR expression and sensitized the cells to the inhibitory effects of 1,25D (80). We also found that inactivation of KLF4 in villin-positive gastric progenitor cells induces the transformation of the gastric mucosa and tumorigenesis in the antrum in mice (81). Villin-Cre(+);KLF4(fl/fl) mice had greater susceptibility to chemical-induced gastric carcinogenesis and higher rates of gastric tumor progression than the control mice. The mouse and human gastric tumors had reduced expression of KLF4 and increased expression of FoxM1 compared with healthy gastric tissue, and expression of KLF4 suppressed the transcription of FoxM1 (81).

### 3.5 VDR and EMT in PDAC CSCs and Drug Resistance

A growing body of evidence suggests that EMT-type cells have CSC characteristics in a variety of human malignancies, including PDAC (5). It is accepted that CSCs, which have been identified using different sets of stem cell surface markers in various types of human cancers, possess the ability to self-renew and generate diverse cell populations (82). To support the link between EMT and CSCs in PDAC, Shah *et al.* found that EMT-type cells have increased expression of the stem cell markers CD24, CD44, and epithelial specific antigen (83). Tsang and Lo revealed that PDAC cells with the EMT phenotype have increased sphere-forming capacity and high expression of CSC surface markers such as CD44 and epithelial cell adhesion molecule (75). Our studies also indicated that the overexpression of VDR by either gene transfection or lentiviral gene transfer caused the downregulation of stem cell markers, including c-Met and CD44, and suppressed the spheroid formation of PDAC cells. In addition, PDAC cells resistant to chemoradiotherapy showed phenotypic and molecular changes consistent with EMT, including increased vimentin and decreased E-cadherin (84). Another study showed that EMT-type cells are resistant to gemcitabine, 5-fluorouracil, and cisplatin, while non-EMT-type cells are sensitive to these chemotherapeutic drugs (85). Furthermore, these resistant cells expressed high levels of the stem cell markers Oct4, CD24, and CD133, indicating that chemoradiotherapy-resistance-induced EMT is associated with CSC generation (84). In a colon cancer study, VDR was found to reverse 5-fluorouracil resistance. To this end, targeting EMT could reduce the population of CSCs that have been implicated in PDAC metastasis and drug resistance (5).

## Summary and Future Directions

The reversal of EMT through the use of VDR has been found to play critical roles in the control of tumor invasion, metastasis, and drug resistance in PDAC. Downregulation of VDR could trigger EMT by several factors, including cytokines and cellular signaling pathways such as β-catenin, FoxM1, and CSCs. More importantly, specific natural compounds could partially reverse the EMT phenotype to mesenchymal-to-epithelial transition, resulting in the reversal of drug resistance. Therefore, targeting the VDR pathway with nontoxic natural agents could be a novel potential therapeutic strategy for the treatment

of metastatic PDAC. However, the molecular mechanisms of EMT are very complicated and are not yet fully elucidated. Therefore, further investigation is necessary to explore the mechanisms underlying EMT progression in PDAC. Future research will surely focus on uncovering the molecular similarities and differences among the EMT programs. EMT research in the next few years promises to be exciting, as new mouse models and molecular probes are identified to address the important, still-unanswered questions.

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## Abbreviations used in this paper

| | |
|---|---|
| **PDAC** | pancreatic ductal adenocarcinoma |
| **EMT** | epithelial-to-mesenchymal transition |
| **1,25D** | 1,25-dihydroxyvitamin D(3) |
| **VDR** | vitamin D receptor |
| **FOXM1** | fork-head box M1 |
| **KLF4** | Krüppel-like factor 4 |
| **CSCs** | cancer stem cells |
| **5-FU** | 5-fluorouracil |

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