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Epigenetic Therapeutic Strategies to Target Molecular and Cellular Heterogeneity in Pancreatic Cancer

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Abstract
Background: Pancreatic ductal adenocarcinoma (PDAC) remains a major challenge in cancer medicine and is characterized by a 5-year survival rate of <10%. Compelling evidence suggests that the devastating disease outcome of PDAC patients is linked to a high degree of intra- and interindividual tumor heterogeneity, which is predominantly installed at the level of gene transcription. The cellular and molecular complexities of the disease explain the poor efficacy of "one-size-fits-all" therapeutic approaches in PDAC treatment and strongly argue for pursuing tailored therapeutic strategies to tackle PDAC. In a highly dynamic manner, a network of transcription factors and epigenetic regulatory proteins temporally and spatially control the diverse transcriptomic states determining PDAC heterogeneity. Given the reversibility of epigenetic processes, pharmacological intervention with key epigenetic drivers of PDAC heterogeneity appeals as a promising concept to shift the transcriptomic phenotype of PDAC toward a less aggressive and more chemosensible state. ***Summary:*** In this review, we discuss the chances and pitfalls of epigenetic treatment strategies in overcoming and shifting molecular and cellular PDAC heterogeneities in order to combat PDAC. To this end, we utilized the keywords "pancreatic cancer," "heterogeneity," and "epigenetics" to search for relevant articles on the database PubMed and selected interventional studies enrolling PDAC patients as displayed in clinicaltrails.gov to generate a synopsis of clinical trials involving epigenetic targeting. ***Key Messages:*** Targeting epigenetic regulators in PDAC represents a promising concept to reprogram molecular and cellular tumor heterogeneities in the pancreas and hence to modulate the PDAC phenotype in favor of a less aggressive and more therapy susceptible disease course. However, we just start to understand the complex interactions of epigenetic regulators in controlling PDAC plasticity, and a clinical breakthrough utilizing epigenetic targeting in PDAC patients has not been achieved yet. Nevertheless, increasing translational efforts which consider the pleiotropic effects of targeting epigenetic regulation in different cellular compartments of the tumor and that focus on the utility and sequence of combinatory treatment approaches might pave the way toward novel epigenetic treatment strategies in PDAC therapy.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) remains a major challenge in cancer medicine. Indeed, despite remarkable efforts in translational research and drug development, the overall 5-year survival rate of <10% of patients has remained unchanged for almost 20 years [1]. Major causes for the dismal outcome are the exceptionally aggressive tumor biology with early onset of metastasis and the remarkable resistance to conventional chemotherapy. Tumor cell progression and therapy evasion

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processes are driven by a high degree of cellular and molecular tumor heterogeneity [2]. Increasing evidence suggests that PDAC heterogeneity is determined by transcriptomic phenotypes, which are hierarchically installed and controlled by epigenetic cues [2–8]. Epigenetic regulatory proteins converge on the transcriptional landscape by controlling, for example, chromatin accessibility, DNA methylation, and histone modification, thus fine-tuning the transcriptional output of a given cell in a spatially and temporally restricted manner [9]. The dynamic nature and the reversibility of epigenetic processes characterize epigenetic regulatory proteins as promising targets to shift transcriptional phenotypes of cancer cells toward less aggressive and more therapy susceptible states. In this review, we discuss conceptual and translational efforts exploiting epigenetic targeting for PDAC treatment with a particular focus on the consequences of epigenetic reprogramming on the molecular and cellular heterogeneity of the disease.

Targeting Epigenetics to Interfere with Molecular PDAC Heterogeneity

Molecular PDAC Subtypes

Given the advances in next-generation sequencing technologies and motivated by the successes of molecular stratification-based treatment approaches in other tumor entities [10–12], the last decade has witnessed a plethora of whole-genome sequencing studies and transcriptional profiling analyses conducted in large cohorts of PDAC tumors aiding at the dissection of the molecular landscape of PDAC [2–8, 13]. These studies did not only reaffirm signature mutations in *KRAS*, *TP53*, *CDKN2A*, and *SMAD4* but led to the identification of numerous additionally mutated or transcriptionally altered genes. Importantly, the molecular heterogeneity of PDAC is reflected in the identification of various molecular and phenotypic PDAC subtypes with prognostic and therapy predictive significance [3–5, 14]. Despite discrepancies in the definition of molecular PDAC subtypes, transcriptome and epigenome analyses consistently identified 2 major lineages which separate PDAC into basal-like (also called "squamous" or "quasi-mesenchymal") and classical (also considered as "progenitor-like") subtypes [2–8, 13, 14]. While the basal-like subtype is associated with a high tumor grade, strong chemoresistance, and the worst prognosis [3–5, 14], classical subtype tumors are better differentiated, associated with improved responsiveness to chemotherapy and a better prognosis [3, 4, 14]. Molecularly, these subtypes are linked to distinct gene signatures and epigenetic profiles [8, 15]. While the basal-like subtype shows a more mesenchymal expression profile, the classical subtype comprises an epithelial differentiation gene signature [2–8, 13, 14]. Moreover, the 2 subtypes differ in the activity of distinct superenhancers and their upstream regulators [8]. Superenhancers operate as regulatory elements known to have a huge ability to influence target gene expression, to have cell- and state-specific activities, and to be bound by lineage-defining transcription factors [16, 17]. The most prominent transcription factors regulating subtype-specific superenhancers and transcription programs are GATA6, PDX1, and HNFs for the classical, and MET, MYC, and the ΔN isoform of the transcription factor TP63 (ΔNp63), for the basal-like state [8, 15, 18–20]. Importantly, compelling evidence suggests that epigenetic regulators complement and control the activity of these subtype-determining transcription factors, either by influencing their expression or by acting as transcriptional co-regulators [20, 21]. Given the dynamic character of these drivers of PDAC subtype identity, the distinct subtype states are not permanently installed but underlay a high degree of plasticity. Considering the better prognosis and the increased chemosensitivity of classical versus basal-like PDAC subtypes [3–5, 14], the concept of subtype switching seems to be a highly appealing strategy, for example, preceding cytotoxic PDAC therapy. Consistent with this idea, many translational approaches in PDAC aim at deciphering strategies to induce subtype switching. Given the reversibility of epigenetic regulations, pharmacological interference with epigenetic key regulators of PDAC subtype identity has moved into the focus of approaches aiding at molecular PDAC heterogeneity.

Epigenetic Targeting Strategies to Induce Subtype Switching in PDAC

Above others, the endodermal lineage transcription factor GATA6 has been characterized as a hierarchical regulator of classical PDAC subtype identity [4, 8, 22] and as a robust surrogate biomarker for differentiating classical ($GATA6^{high}$) and basal-like ($GATA6^{low}$) PDAC subtypes [14]. Consistently, depletion of *GATA6* is necessary to induce the basal subtype-specific transcription factor ΔNp63 and to enforce a basal-like PDAC subtype state [22]. Hence, pharmacological approaches inducing or stabilizing GATA6 expression qualify as promising strategies to push PDAC cells toward the classical PDAC subtype. The potential of GATA6 induction for enforcing classical PDAC subtype identity was introduced by a study of Lomberk et al. [8]. Herein, the authors demonstrate that inhibition of the basal-like subtype-specific superenhancer regulator MET induces a basal-like-to-classical subtype switch via transcriptional activation of *GATA6* and subsequent induction of GATA6-dependent gene regulation [8]. Along the same line of evidence, loss of the basal-like subtype-specific transcription factor GLI2, which is involved in Hedgehog signaling [23], re-

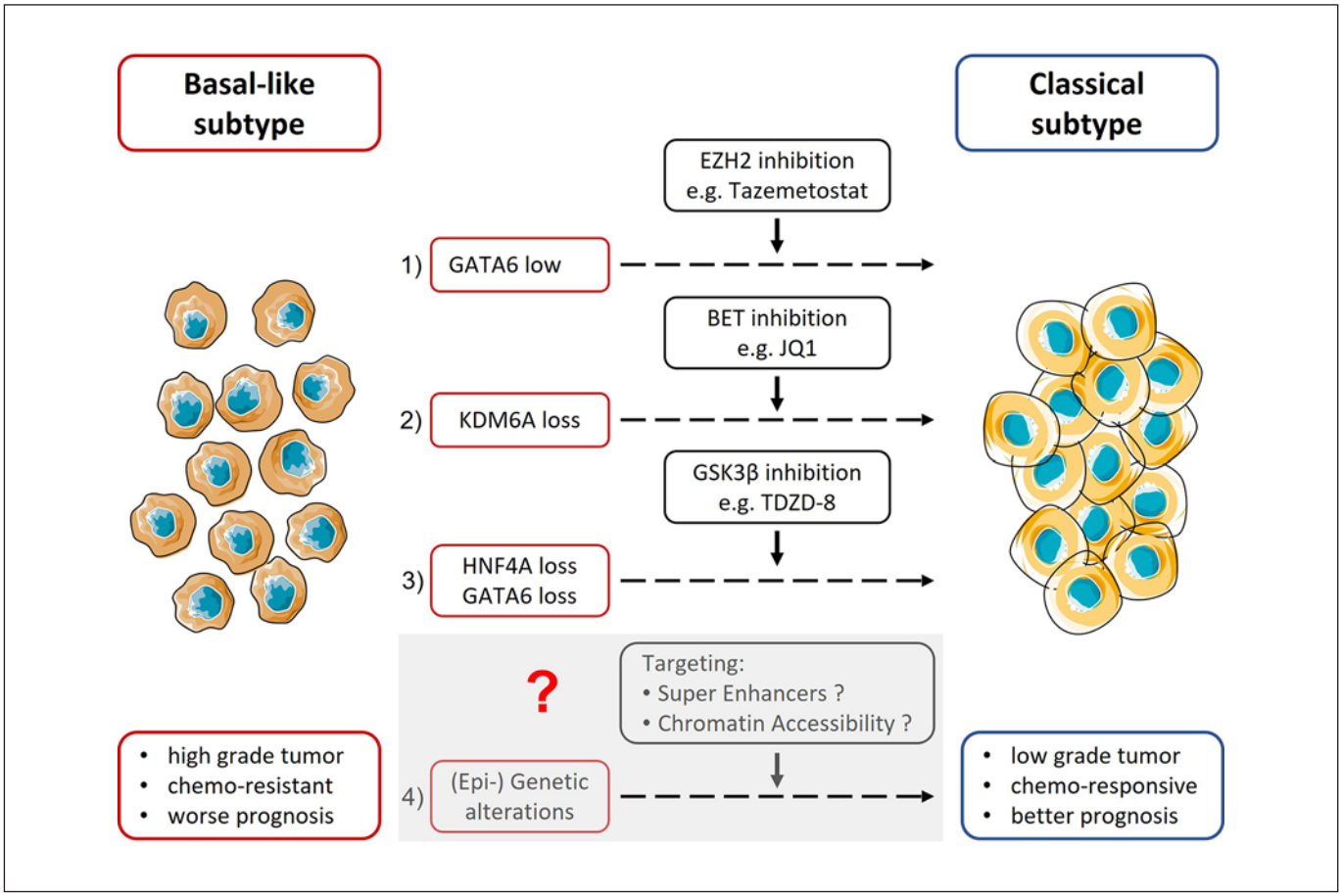

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Fig. 1. Epigenetic targeting strategies to tackle PDAC subtype identity. PDAC can be classified into the classical and basal-like subtypes differing in chemosensitivity and prognosis. However, there is a dynamic plasticity between these 2 PDAC subtypes. Therefore, forcing a subtype switch from the aggressive basal-like subtype into the less aggressive classical subtype might be a compelling therapeutic option. (1) Derepression of *GATA6* expression upon EZH2 inhibition (e.g., using tazemetostat) [21]; (2) BET inhibition (e.g., using JQ1) in KDM6A-deficient PDAC [20]; and (3) GSK3β inhibition (e.g., using TDZD-8) PDAC subtypes characterized by low expression of GATA6/HNF4A [26]. (4) Prospectively, it can be hypothesized that targeting superenhancer activity or chromatin accessibility might be an appealing approach to target PDAC subtype identity upon molecular stratification of the tumor [8, 26, 57]. Designed with https://smart.servier.com/. PDAC, pancreatic ductal adenocarcinoma; BET, bromodomain and extra-terminal motif; DNMT, DNA methyltransferase.

sults in increased *GATA6* expression and acquisition of classical gene signatures in PDAC [24]. While these reports emphasize the role of subtype-specific transcription factors in regulating the expression of their classical counterpart *GATA6*, Patil et al. [21] recently identified the histone methyltransferase enhancer of zeste homolog 2 (EZH2) as a direct transcriptional regulator of *GATA6* in PDAC. In accordance with its activity as a transcriptional repressor, EZH2 binding to the *GATA6* TSS region silenced *GATA6* transcription, thus promoting PDAC invasion and metastasis. Interestingly, blockade of EZH2 activity was sufficient to reinstall *GATA6* expression and to induce gene signatures associated with the classical PDAC subtype state. Hence, these data reveal pharmacological interference with EZH2-dependent *GATA6* repression as a promising strategy to induce subtype switching, restrain tumor progression, and increase chemosensitivity in PDAC (Fig. 1) [21]. However, given the existence of PDAC tumors with EZH2-independent GATA6 regulation [1], EZH2 targeting might only be beneficial in a subgroup of GATA6^{low} PDAC subtypes, thus arguing for molecular stratification of *GATA6* expression prior to application of EZH2 inhibitors. Nevertheless, the recent FDA approval of the EZH2 inhibitor tazemetostat for the treatment of advanced epithelioid sarcoma and a clinical trial exploring the drug in solid tumor entities including PDAC (NCT04705818, Table 1) suggest a potential clinical relevance of the identified EZH2-GATA6 axis in PDAC (Fig. 1).

In addition to a simultaneous loss of GATA6, epigenetic silencing of *HNF4A* and *HNF1A*, has been lately reported as a prerequisite for basal-like subtype identity

Table 1. Currently active clinical trials involving epigenetic treatment strategies in PDAC

NCT number	Status	Drug	Co-treatment	Target	Phase	Enrolled tumor entities
NCT03264404	Recruiting	Azacitidine	Pembrolizumab	DNMT1	2	Pancreatic cancer
NCT01845805	Active, not recruiting	Azacitidine	Abraxane, gemcitabine	DNMT1	2	Pancreatic cancer
NCT04257448	Recruiting	Azacitidine, romidepsin	Nab-paclitaxel + gemcitabine, durvalumab + lenalidomide capsule	DNMT1, HDAC class I	1/2	Pancreatic cancer
NCT03250273	Active, not recruiting	Entinostat	Nivolumab	HDAC class I	2	Pancreatic cancer, cholangiocarcinoma
NCT01638533	Active, not recruiting	Romidepsin	None	HDAC class I	1	Pancreatic cancer and other solid tumor entities, hematological malignancies
NCT04705818	Not yet recruiting	Tazemetostat	Durvalumab	EZH2	2	Pancreatic cancer and other solid tumor entities
NCT02349867	Active, not recruiting	Vorinostat	Gemcitabine + sorafenib + chemoradiation	HDAC class I/II	1	Pancreatic cancer
NCT03878524	Recruiting	Vorinostat	52 drugs (chemotherapy, small inhibitors, antibodies)	HDAC class I/II	1	Pancreatic cancer and other solid tumor entities, hematologic malignancies

PDAC, pancreatic ductal adenocarcinoma; DNMT, DNA methyltransferase; HDAC, histone deacetylase. Selection criteria applied in clinicaltrails.gov: interventional study type, pancreatic cancer, epigenetic treatment involved.

[22]. Loss of HNF4A favors upregulation of GSK3β, which promotes metabolic programs in accordance with the basal-like PDAC subtype [25]. Interestingly, basal-like PDAC subtypes were more sensitive to pharmacological inhibition of GSK3β (Fig. 1) [26]. However, the fact that a subgroup of PDAC developed resistance toward GSK3β inhibition [26] suggests the existence of subgroups within the basal-like PDAC subtype state, whose therapeutic utilities remain to be further explored.

Subtype switching induced by interfering with the epigenetic landscape in PDAC has recently been reported for a subgroup of basal-like PDAC subtypes harboring mutations in the gene encoding for the histone demethylase KDM6A. Mechanistically, loss of KDM6A activity results in aberrant activation of superenhancers that regulate the *ΔNp63*, *RUNX*, and *MYC* oncogenes, thus fostering dedifferentiation and metastasis [20]. Importantly, the increased superenhancer activity renders *KDM6A*-deficient PDAC more susceptible to inhibition of bromodomain and extra-terminal motif (BET) proteins, which reverses basal-like differentiation and restrains PDAC growth both in vitro and in vivo (Fig. 1) [20]. Using preclinical PDAC models, BET-protein inhibition combined with blockade of histone deacetylase (HDAC) activity has been previously introduced as a promising concept in PDAC treatment [27], albeit without considering the PDAC subtype state. Hence, the stratification for *KDM6A* might even increase the efficacy of BET inhibition for PDAC treatment.

Together, interference with epigenetic regulators to target subtype-specific pathways and expression profiles offers strategies to conquer drug resistance and improve the outcome of PDAC patients. However, the safe and effective application of epigenetic targeting strategies to interfere with PDAC subtype identity requires a more detailed picture reflecting the entire complexity of PDAC molecular heterogeneity. Moreover, regulation of molecular PDAC subtype identity is not restricted to the epithelial tumor cell but is highly influenced by the interaction with other cellular compartments of the tumor.

Targeting Epigenetics to Interfere with Cellular PDAC Heterogeneity

The cellular heterogeneity of PDAC is based on a pronounced tumor microenvironment (TME) that already forms during pancreatic carcinogenesis and evolves during tumor progression [28]. The PDAC TME, which makes up to 90% of the tumor bulk, comprises cellular (e.g., fibroblasts and immune cells) and acellular components (e.g., collagen and hyaluronic acid), and intensive biochemical interactions exist between these different compartments and the epithelial tumor cells [29, 30]. Im-

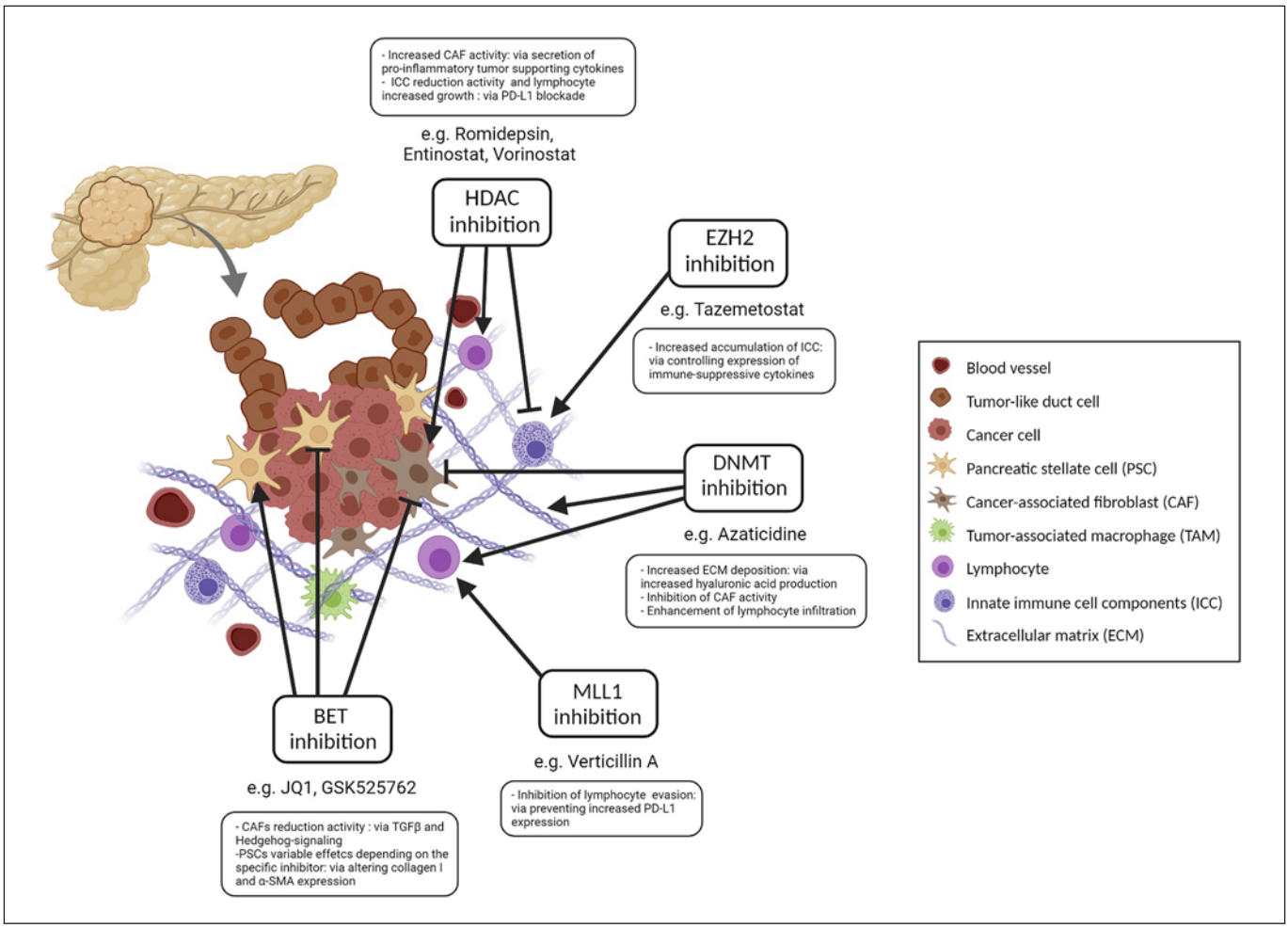

Fig. 2. Epigenetic targeting strategies of the PDAC TME. The main challenge in utilizing epigenetic therapeutic strategies to tackle the cellular heterogeneity of PDAC is associated with the complex and multifactorial role of epigenetic regulation occurring in the different cellular PDAC compartments. Consequently, inhibition of epigenetic regulators can shift the cellular composition of the TME toward a more or less tumor promoting stroma composition. The cartoon was created with BioRender.com. PDAC, pancreatic ductal adenocarcinoma; BET, bromodomain and extra-terminal motif; TME, tumor microenvironment; PSC, pancreatic stellate cell; CAF, cancer-associated fibroblast; TAM, tumor-associated macrophage; ICC, innate immune cell components; ECM, extracellular matrix; HDAC, histone deacetylase.

portantly, the TME does not only promote PDAC progression, but it also significantly impacts on PDAC therapy response. Indeed, acellular and cellular components of the PDAC stroma significantly reduce the exposure of tumor cells to chemotherapeutic agents, for example, by causing a hypovascular microenvironment and by scavenging of active chemotherapeutic metabolites, respectively [29, 31–33]. Moreover, the immune cell components of the PDAC stroma do not eliminate, but rather tolerate tumor cells [34, 35]. Despite its implication for PDAC progression and therapy resistance, therapeutic efforts aiding at stroma depletion have not proved successful in combatting PDAC [28]. Rather, the strong plasticity of the PDAC TME argues for reprogramming the PDAC stroma in favor of a less aggressive and more therapy susceptible TME. Not surprisingly, epigenetic mechanisms play a pivotal role in controlling the dynamic plasticity of the cellular components of the PDAC stroma and hence represent promising targets to interfere with the cellular heterogeneity of the disease.

Epigenetic Treatment Strategies to Target the PDAC TME

Several studies have investigated the role of DNA methylation processes in the regulation of gene expression in multiple components of the TME. For instance, blockade of DNA methylation by applying the DNA methyltransferase (DNMT) inhibitor 5-azacytidine reduced PDAC progression by interfering with global DNA methylation in epithelial PDAC cells and cancer-associated fibroblasts (CAFs) (Fig. 2) [36]. Further supporting the utility of DNMT inhibition in PDAC TME repro-

gramming, DNMT blockade in immunocompetent PDAC models enhanced $CD4^+$ and $CD8^+$ T-cell infiltration and caused significant tumor regression (Fig. 2) [37]. However, in contrast to these findings, pharmacological or genetic depletion of DNMT1 resulted in increased production of hyaluronic acid, thus promoting PDAC progression (Fig. 2) [38]. A correlation of low DNA methylation levels and poor PDAC patient outcome has recently also been reported by Espinet et al. [39]. They demonstrate that tumors with a low global DNA methylation in the epithelial cells are characterized by a higher expression of endogenous retroviral transcripts and a strong engagement of the double-stranded RNA sensing machinery with subsequent activation of an interferon signature, thus resulting in pro-tumorigenic reprogramming of stromal cells in the PDAC TME [39]. These contrary findings on the role of DNA methylation in different cellular compartments of the TME suggest that balancing epigenetic treatment strategies is very crucial to find the optimal antitumor composition of epigenetic drugs.

Similar to the implication in driving molecular PDAC subtype identity [8, 15], enhancers play a pivotal role in PDAC TME cell specification. Indeed, enhancers have been characterized as "entry gates" for external signaling cues that promote transcription programs required to establish and maintain a TME composition, which fosters tumor development and progression [28, 40, 41]. BET proteins represent critical cofactors that promote (super)enhancer activity and play a pivotal role in PDAC progression, not only in epithelial tumor cells but also in the PDAC stroma [27, 42, 43]. Consequently, BET inhibition decreases TGFβ and hedgehog signaling, thus reducing the activity of CAFs (Fig. 2), which harbor significant implications in promoting PDAC progression and therapy resistance [28, 44]. It is worth noticing that the various BET inhibitors available for clinical or preclinical studies do have distinct specificities for the different BET protein family members BRD2, 3, and 4 [45, 46]. Given that BRD4 and BRD2/3 promote and block collagen I expression in pancreatic stellate cells, respectively [47] (Fig. 2), a careful selection of these inhibitors based on their target BET protein specificity might be of particular clinical implication. In addition to selecting the right BET inhibitor to combat PDAC, BET inhibition seems to be particularly efficient when combined with the standard chemotherapeutic agent in PDAC treatment, gemcitabine [27, 48], or other inhibitors of epigenetic regulators. The combination with HDAC inhibitors, for instance, showed very promising results in preclinical PDAC models [27], although HDAC inhibition in PDAC per se has been linked to the secretion of pro-inflammatory tumor-supporting cytokines by CAFs, thus promoting a tumor-supportive phenotype (Fig. 2) [49]. These findings emphasize the complexity of epigenetic targeting strategies in general, and in remodeling the pancreatic stroma, in particular. With regard to targeting HDAC proteins, this complexity is even increased given the number of HDAC protein family members and their respective diverse functional involvements [50]. For instance, HDAC proteins are not only mediating HDAC and hence transcriptional regulation but also interfere with posttranslational acetylation of a plethora of target proteins [51]. Consequently, the global and partially unpredictable effects of the diverse HDAC proteins and, therefore of their inhibition, represent a significant obstacle for HDAC inhibition in PDAC treatment. Hence, and in the interest of higher drug specificities, currently active clinical trials exploring HDAC inhibition in PDAC treatment concentrate on HDAC class I and II specific inhibitors (Table 1).

EZH2 is another chromatin regulatory protein, which directs TME-reprogramming processes in PDAC. Interestingly, and as illustrated in *Ezh2*-deficient and *Kras*-mutant transgenic mouse models of PDAC, conditional *Ezh2* deficiency resulted in increased accumulation of CD11b+ macrophages, Gr-1+ neutrophils, and CD11c+ dendritic cells in the pancreas, indicating a higher recruitment of innate immune system players (Fig. 2) [52, 53]. These shifts in the immune compartment of the TME were accompanied by increased collagen deposition and αSMA expression and strongly promoted pancreatic carcinogenesis [52]. Given that the ablation of *Ezh2* in this model specifically occurred in the epithelial, but not in the stromal cells, these data further imply a strong communication between the epithelial and the TME cellular components of the pancreas. This note is further supported by the fact that the application of the Cox2 inhibitor nimesulide rescued the inflammatory response in these mice and prevented the formation of advanced PDAC precursor lesions [52]. The observations made in the *Ezh2*-deficient transgenic model are accomplished by several reports, indicating the critical involvement of epigenetic players in controlling immune cell regulation in PDAC development in progression. For instance, epigenetic mechanisms impact on antigen processing and presentation by tumors cells, control the transcription of immune-suppressive cytokines, and impair cytotoxic T-cell function [54]. Accordingly, the *CD274* gene, encoding for the immune checkpoint inhibitor PD-L1, is heavily enriched for the H3K4me3 histone mark, rendering its promoter transcriptionally active. This histone mark is installed by the methyltransferase MLL1. Consequently, MLL1 inhibition has been reported to partially prevent increased PD-L1 expression and subsequent immune cell evasion [55]. A similar efficacy in overcoming immune cell evasion in PDAC has been shown upon combining the checkpoint inhibitors anti-PD-1 or anti-CTLA-4 with HDAC inhibition. This combinatory treatment strategy led to a significant abundance of cytotoxic T cells by decreasing the activity of

myeloid-derived suppressor cells in the TME (Fig. 2) [56]. This report strongly argues for therapeutic strategies in which epigenetic treatment concepts are utilized to prime the immune PDAC TME for the subsequent exposure to immunomodulatory drugs. A sequential approach based on HDAC/DNMT inhibition combined with chemotherapy and followed by PD-L1 blockade is currently undergoing clinical testing in PDAC (NCT04257448, Table 1), and results are eagerly awaited.

Conclusion

Without doubt, epigenetic regulatory proteins represent pivotal drivers of PDAC tumor progression and therapy resistance, not only, but particularly by determining the cellular and molecular heterogeneity of the disease. Translational studies conducted within the last years have convincingly demonstrated the utility of epigenetic targeting in interfering with PDAC plasticity and switching between different cellular and molecular states. Nevertheless, in contrast to other tumor entities, epigenetic targeting of PDAC is still in its infancy. This is also indicated by the relatively low number of epigenetic drugs which qualify for clinical testing in PDAC (Table 1). Despite significant efforts, important technical advances and the increasing understanding of the implications of PDAC heterogeneity for the disease course and outcome, we have only started to dissect the molecular underpinnings and the upstream regulatory components, which finally give rise to the complex epigenetic landscape evident in PDAC. The dynamic nature of epigenetic alterations allows reverting cellular states and conditions in favor of a less aggressive and more therapy susceptible molecular and cellular makeup of PDAC. However, epigenetic mechanisms often play contrary roles in different cellular components of PDAC, with tumor-promoting activities in one, and tumor-suppressive functions in other compartments. Unfortunately, current epigenetic targeting strategies cannot distinguish between the different functional implications of spatially distinct but otherwise identical, epigenetic mechanisms.

In contrast to hematological malignancies in which epigenetic treatment strategies play an increasing role even in monotherapeutic application, it does not seem likely that targeting of 1 epigenetic regulator or even of a family of epigenetic proteins is sufficient to combat PDAC. As described in this article, first promising results have been reported for combinatory treatment strategies, which involve epigenetic targeting. From these studies, we gained first evidence that epigenetic therapies have a strong potential in priming PDAC tumor cells and their TME for additional, predominantly cytotoxic, therapy. Hence, in addition to further disentangle the complex interactions between epigenetic mechanisms and additional regulatory processes evident in PDAC, translational efforts need to unravel the best sequence of epigenetic drug application to combat this dismal disease.

Conflict of Interest Statement

The authors have no conflict of interest to declare.

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Author Contributions

L.V. wrote the part on epigenetics in PDAC molecular heterogeneity and drafted Figure 1. M.U. wrote the part on PDAC cellular heterogeneity and designed Figure 2 and Table 1. E.H. developed the review's concept and structure, wrote the introduction and the conclusion, and compiled all sections of this review.

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