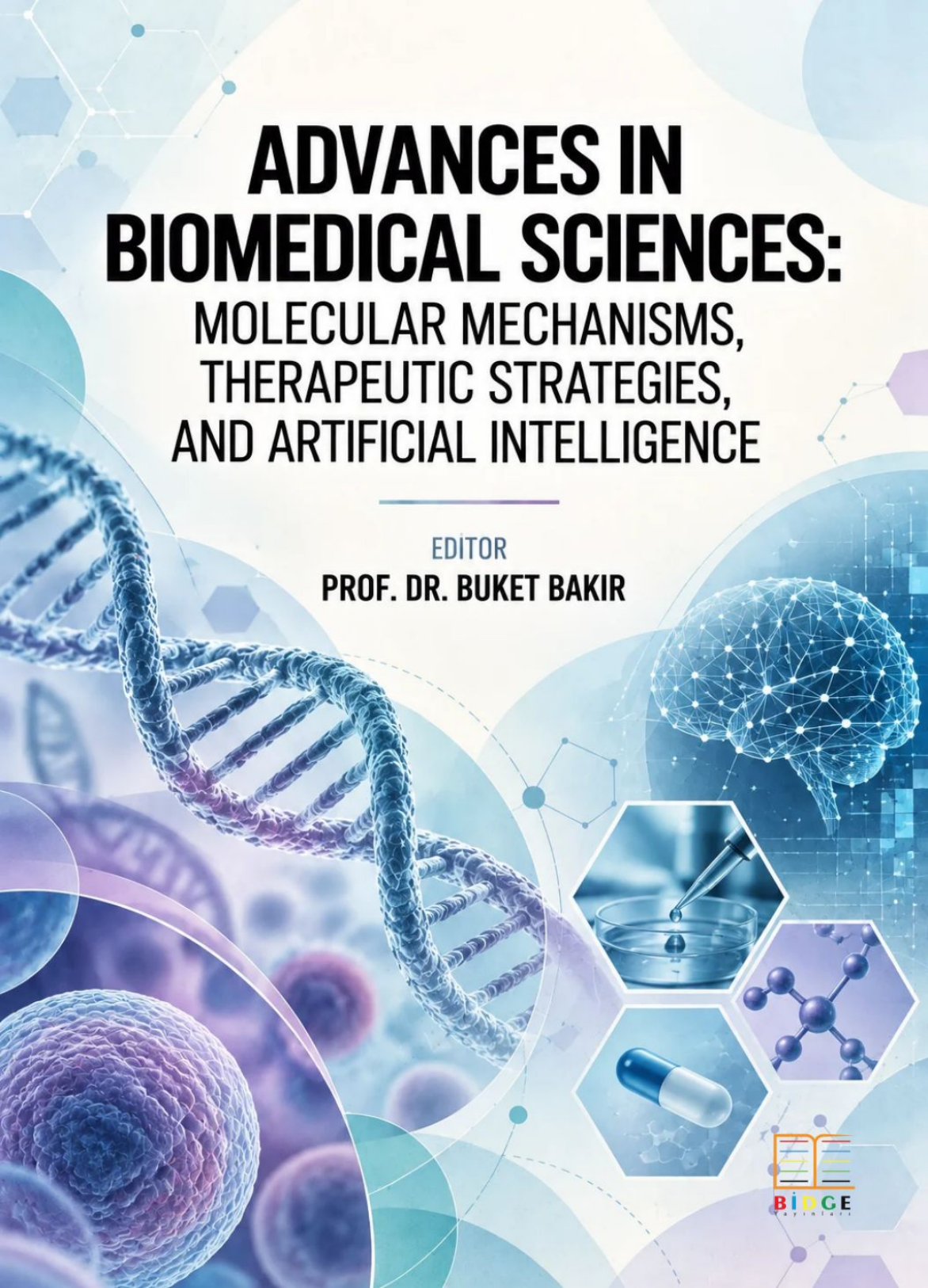
ADVANCES IN BIOMEDICAL SCIENCES:

MOLECULAR MECHANISMS, THERAPEUTIC STRATEGIES, AND ARTIFICIAL INTELLIGENCE

EDITOR

PROF. DR. BUKET BAKIR



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CHAPTER 1

NEURAL REGULATION OF PANCREATIC CANCER: NEUROTRANSMITTER SIGNALING AND THE REPURPOSING OF CNS-ACTING DRUGS

Buse BİRİNCİ GENÇ[1], Sabire GÜLER[2]

Introduction

Pancreatic ductal adenocarcinoma (PDAC), representing the vast majority of pancreatic malignancies (Jandaghi et al., 2016), remains among the most lethal solid cancers. Surveillance figures identify pancreatic cancer as the third leading cause of cancer death in the United States (Siegel et al., 2026), realizing earlier epidemiological projections that it would become the second by 2030 (Rahib et al., 2014). Late clinical detection remains the primary obstacle to curative resection; despite improvements in surgical technique and multi-agent chemotherapy regimens, most patients present with advanced disease, underscoring the urgent need for non-canonical therapeutic approaches.

A defining characteristic of PDAC is its fibroinflammatory, desmoplastic stroma, which forms a nutrient-deprived, hypovascular, yet densely innervated microenvironment (Banh et al., 2020). Over the past decade, cancer neuroscience has transformed our understanding of the peripheral nervous system, showing that innervating fibers actively drive tumor initiation and progression rather than remaining passive bystanders (Monje et al., 2020). Pancreatic cancer provides a clear paradigm of this neuro-epithelial relationship. Perineural invasion—the process whereby malignant cells invade and spread along peripheral nerves—represents a hallmark histopathological feature that facilitates local dissemination and causes severe neuropathic pain (Bapat et al., 2011; Liebig et al., 2009). Intrapancreatic nerve remodeling begins early in pathogenesis; structural neuroplastic alterations within sensory and autonomic circuits are already detectable during pre-invasive stages (Stopczynski et al., 2014).

Interactions between peripheral nerves and cancer cells are bidirectional and molecularly diverse. Differentiated Schwann cells actively track neoplastic clones, promoting their detachment and directional migration along axonal fibers through *NCAM1* signaling (Deborde et al., 2016). Sympathetic signaling via the β2-adrenergic receptor establishes a feed-forward circuit with tumor-derived nerve growth factor (NGF), expanding intratumoral nerve density and accelerating tumor growth (Renz, Takahashi, et al., 2018). Conversely, disrupting parasympathetic input by subdiaphragmatic vagotomy accelerates tumor progression and shortens survival in murine models (Partecke et al., 2017). Sensory neurons form functional glutamatergic pseudo-synapses with

[1] M.Sc. Student, Bursa Uludağ University, Institute of Health Sciences, Department of Veterinary Histology and Embryology, ORCID: 0009-0007-2150-9868
[2] Assoc. Prof. Dr., Bursa Uludağ University, Faculty of Veterinary Medicine, Department of Histology and Embryology, ORCID: 0000-0002-7367-6859

carcinoma cells, creating an electrochemical link through which neural activity drives tumor growth (Ren et al., 2025). Axons also provide metabolic support: within the nutrient-depleted stroma, peripheral nerves release free serine into the extracellular space to sustain neoplastic protein translation (Banh et al., 2020). Recent single-cell and spatial transcriptomic profiling has mapped the distinct cellular states involved in this neural invasion (Chen et al., 2025).

Because these pathways operate through neurotransmitter signaling, they offer direct opportunities for drug repurposing. Centrally acting neurotherapeutics have well-established safety profiles, defined clinical pharmacokinetics, and generic availability, bypassing early-phase development hurdles. Antagonizing the dopamine D2 receptor (DRD2) suppresses tumor expansion in preclinical PDAC models (Jandaghi et al., 2016). The histamine H1 receptor (*HRH1*) has likewise emerged as a candidate target responsive to approved antihistamines (Insel et al., 2026; Salmerón et al., 2025). Pharmacological blockade of HRH1 upregulates major histocompatibility complex (MHC) class I presentation on pancreatic cancer cells, augmenting immune checkpoint therapy (Zhong et al., 2024). Benztropine, an anticholinergic approved for Parkinson's disease, exemplifies both the therapeutic potential and mechanistic complexity of this strategy. The compound suppresses breast cancer stem cells (Cui et al., 2017) and inhibits tumor growth and metastasis through the dopamine transporter (*SLC6A3*) (Sogawa et al., 2020). However, pharmacological binding databases reveal that benztropine binds both the muscarinic M1 receptor (*CHRM1*) and histamine H1 receptor (*HRH1*) with sub-nanomolar affinity, whereas its affinity for the dopamine transporter (DAT, encoded by *SLC6A3*) is several hundred-fold lower (Zdrazil et al., 2024). Determining which of these candidate targets is functionally present in human PDAC is an essential, unresolved question.

In this chapter, we synthesize current knowledge on the neural microenvironment of PDAC, examining cholinergic, adrenergic, sensory, dopaminergic, and histaminergic signaling. We evaluate the clinical and preclinical evidence supporting the repurposing of CNS-active drugs. To resolve conflicting mechanistic hypotheses, we integrate public transcriptomic datasets from bulk patient cohorts, normal pancreas controls, cell lines, and spatial transcriptomics. This exploratory analysis examines whether candidate neuroreceptors are expressed in human PDAC, identifies their cellular compartments, and tests whether their expression tracks with anatomical nerve proximity, molecular subtype, or patient survival.

The Neural Microenvironment of Pancreatic Cancer

The pancreas receives rich autonomic and sensory innervation. Parasympathetic fibers arrive via the vagal trunk, sympathetic projections course through the splanchnic nerves and celiac ganglion, and primary sensory afferents run in parallel. Under physiological conditions, these fibers coordinate endocrine and exocrine secretion; upon disease onset, they undergo striking structural changes. Both chronic pancreatitis and PDAC exhibit neural hypertrophy and increased nerve density, an altered autonomic-to-sensory fiber balance, and endoneurial infiltration by inflammatory or neoplastic cells (Demir et al., 2015). These neural alterations are not simply late consequences of tumor growth. In genetically engineered mouse models, sensory neuroinflammation and axon damage are detectable at the stage of pancreatic intraepithelial neoplasia, with neoplastic cells migrating along sensory nerves long before invasive carcinoma is established (Saloman, Albers, Li, et al., 2016; Stopczynski et al., 2014).

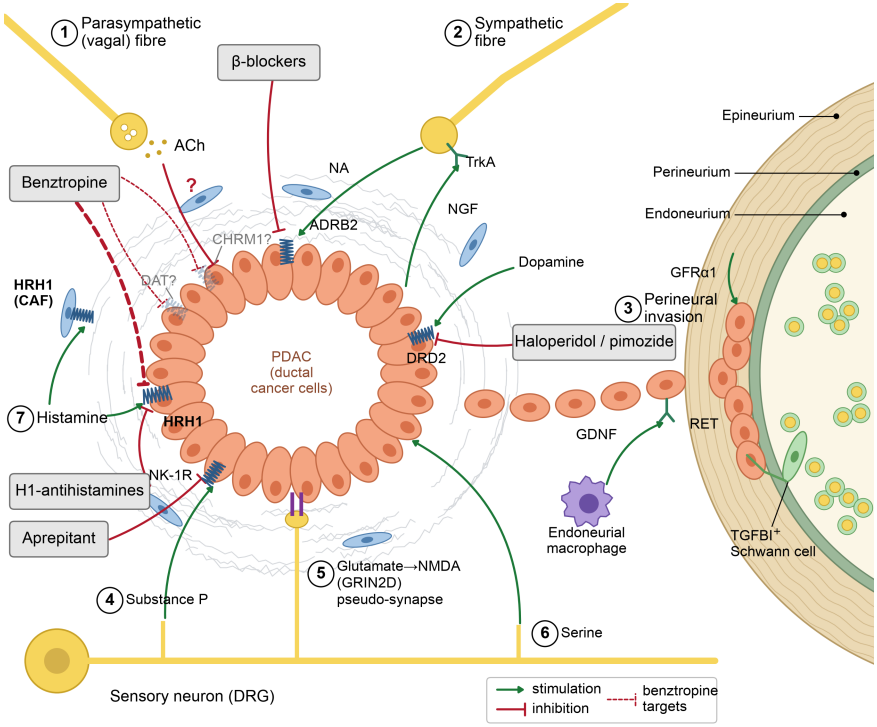
Perineural invasion represents the pathological hallmark of this neuro-tumoral relationship. Histopathologically, perineural invasion is defined as tumor cell presence within any of the three layers of the nerve sheath—the epineurium, perineurium, or endoneurium—or tumor cell abutment covering at least 33% of a nerve's circumference (Liebig et al., 2009). This standardized threshold

differentiates true nerve invasion from incidental proximity. Standard hematoxylin and eosin sections detect gross invasion, with S100 immunohistochemistry aiding the visualization of small nerve twigs. Observed in the majority of surgically resected PDAC specimens, perineural invasion drives local retroperitoneal recurrence and correlates with severe neuropathic pain (Bapat et al., 2011; Cavel et al., 2012). A meta-analysis of over one hundred studies confirmed that neural invasion serves as an independent prognostic factor for overall, disease-free, and progression-free survival (Schorn et al., 2017).

Perineural invasion proceeds via active chemotactic guidance rather than passive cell movement along mechanical paths of least resistance. Schwann cells directly contact cancer cells, intercalating into tumor nests to promote cell dispersion and directed migration toward nerves through *NCAM1* signaling (Deborde et al., 2016). Neurotrophic factors provide directional guidance: nerve growth factor (NGF) signaling via TrkA and p75NTR stimulates the migration of pancreatic cancer cells toward dorsal root ganglia, and pathway inhibition reduces neural invasion in vitro (Bapat et al., 2016). In genetically engineered mice, systemic NGF depletion initiated after disease onset decreases neural inflammation, perineural invasion, and metastasis (Saloman et al., 2018). Recruited endoneurial macrophages release glial cell line-derived neurotrophic factor (GDNF), which activates the RET tyrosine kinase receptor on cancer cells to induce directional migration (Cavel et al., 2012). Soluble GFRα1 released from peripheral nerves further amplifies GDNF–RET signaling, conferring responsiveness even to cancer cells that lack endogenous GFRα1 (He et al., 2014).

High-resolution spatial profiling has clarified the multicellular organization of this niche. In an integrated single-cell and spatial transcriptomic study of 25 PDAC patients, tertiary lymphoid structures were abundant in tumors with low neural invasion and clustered near non-invaded nerves, whereas nerves in high-invasion tumors were surrounded by NLRP3-positive macrophages and cancer-associated myofibroblasts (Chen et al., 2025). That study identified a TGFBI-positive Schwann cell subpopulation at the leading edge of neural invasion, induced by transforming growth factor-β (TGF-β) signaling and associated with reduced survival. Malignant cell populations also stratify by invasiveness: basal-like and distinct neural-reactive transcriptional states display heightened affinity for peripheral nerves (Chen et al., 2025). These findings position nerves at the center of an interactive neural-immune-stromal niche (Figure 1), indicating that tumor-intrinsic molecular programs influence neurotropic spread.

Figure 1. Schematic overview of the neural microenvironment of pancreatic ductal adenocarcinoma (PDAC).



(1) Parasympathetic vagal projections release acetylcholine (ACh), proposed to suppress PDAC via the muscarinic M1 receptor (CHRM1); the question mark indicates low CHRM1 transcript expression in human PDAC and uncertain preclinical evidence. (2) Sympathetic fibers release noradrenaline (NA), acting on the β2-adrenergic receptor (ADRB2) on cancer cells; nerve growth factor (NGF) released by cancer cells acts on TrkA on nerve fibers to promote axon growth, establishing a feed-forward loop. (3) Perineural invasion: cancer cells invade the epineurium and spread along the perineurium, guided by Schwann cells, macrophage-derived glial cell line-derived neurotrophic factor (GDNF) signaling through RET, and axon-derived GFRα1. (4–6) Sensory neurons release substance P targeting neurokinin-1 receptors (NK-1R), form glutamatergic pseudo-synapses with NMDA receptors (GRIN2D), and supply serine. (7) Histamine acts on the histamine H1 receptor (HRH1), expressed by both cancer cells and cancer-associated fibroblasts (CAFs). Shaded boxes denote approved drugs targeting each pathway; dashed lines indicate the multiple targets of benztropine (CHRM1, HRH1, and the dopamine transporter DAT), with the bold line highlighting HRH1, the target most consistently expressed in our PDAC analysis.

Source: Created by the authors.

Cholinergic (Parasympathetic) Signaling

Parasympathetic input to the pancreas is delivered mainly by the vagus nerve, and experimental models suggest that it restrains PDAC. In a murine orthotopic model, subdiaphragmatic vagotomy promoted tumor growth and shortened survival in a tumor necrosis factor-α (TNF-α)-dependent manner (Partecke et al., 2017). Using genetically engineered *Kras*-mutant mice, Renz and colleagues reported that vagotomy accelerated tumor development, whereas the muscarinic agonist bethanechol restored normal tissue architecture and prolonged survival in established tumors; these effects were attributed to the muscarinic M1 receptor (*CHRM1*), which suppressed EGFR/MAPK and PI3K/AKT signaling and depleted cancer stem cells (Renz, Tanaka, et al., 2018). An editorial Expression of Concern was formally published for this article in August 2026 ("Editor's Note," 2026), warranting caution until the underlying data are resolved. Retrospective clinical findings lend partial support: in a large cohort of US veterans, use of cholinergic agonists was associated with earlier disease stage at diagnosis, though not with longer overall survival (Cham et al., 2026).

These observations create an apparent paradox for antimuscarinic drugs such as benztropine. If muscarinic M1 signaling suppresses PDAC, an M1 antagonist would be expected to be neutral or

detrimental rather than beneficial. Two pharmacological considerations help resolve this paradox. Benztropine is not a selective antimuscarinic: it binds the histamine H1 receptor with sub-nanomolar affinity and inhibits dopamine reuptake through the dopamine transporter at higher concentrations (Zdrazil et al., 2024). Whether human PDAC tissue expresses *CHRM1* at meaningful levels has rarely been evaluated directly in patient specimens. Our computational analysis addresses this expression question directly.

Adrenergic (Sympathetic) Signaling

Chronic stress and sympathetic tone have been linked to cancer progression across multiple organs. In murine PDAC, catecholamines promote β2-adrenergic receptor (ADRB2)-dependent tumor development, stimulating neoplastic NGF secretion and increasing pancreatic nerve density to establish an adrenergic-neurotrophin feed-forward circuit (Renz, Takahashi, et al., 2018). Sympathetic signaling also impairs antitumor immunity. In a chronic stress model, noradrenaline release induced functional T-cell exhaustion; treatment with the non-selective β-blocker carvedilol reversed this exhausted phenotype and, when co-delivered with an anti-TIM-3 antibody, restored checkpoint immunotherapy responses (W. Li et al., 2026).

These findings generated clinical interest in repurposing β-blockers, but observational evidence remains mixed. In a Norwegian registry study, non-selective β-blocker users showed lower pancreatic cancer-specific mortality compared with patients on other antihypertensives, whereas selective β-blockers showed no association with mortality (Støer et al., 2021). A retrospective cohort of advanced PDAC patients observed no survival benefit associated with β-blocker exposure (Le Bozec et al., 2023). A 2026 meta-analysis across nine retrospective cohorts encompassing over 30,000 patients found no overall association between β-blocker use and all-cause or cancer-specific mortality, but non-selective β-blockers were associated with reduced cancer-specific mortality in subgroup analyses (R. Li et al., 2026). In the US veterans cohort, β-blocker use correlated with earlier stage at diagnosis and improved overall survival (Cham et al., 2026). Together, these findings suggest that clinical benefit is confined to non-selective agents and specific patient subsets, a premise that requires prospective validation.

Sensory, Glutamatergic and Dopaminergic Signaling

Sensory neurons influence cancer biology beyond transmitting pain signals. Chemical ablation of sensory neurons with neonatal capsaicin in genetically engineered mice prevented perineural invasion, delayed pre-invasive lesion formation, and prolonged survival (Saloman, Albers, Li, et al., 2016). Denervation across animal models consistently slows tumor initiation (Saloman, Albers, Rhim, & Davis, 2016). Sensory neurons communicate with cancer cells through neuropeptides. The substance P/neurokinin-1 receptor (NK-1R) axis mediates reciprocal signaling among PDAC cells, sensory neurons, and Schwann cells; in human 3D co-cultures, the approved NK-1R antagonist aprepitant reduced the neural invasion of MIA PaCa-2 cells and enhanced paclitaxel cytotoxicity (Schiedlauske et al., 2026).

Sensory neurons can assemble direct glutamatergic pseudo-synapses with pancreatic ductal adenocarcinoma cells, creating an electrochemical route through which neuronal firing stimulates tumor progression (Ren et al., 2025). Peripheral axons also provide metabolic support: within the nutrient-poor stroma, innervating nerves release free serine into the extracellular space to sustain neoplastic mRNA translation (Banh et al., 2020).

Dopaminergic signaling represents another candidate axis. Dopamine D2 receptor (DRD2) levels are elevated in human PDAC compared with normal tissue. Both genetic knockdown of *DRD2*

and pharmacological blockade with the antipsychotics pimozide and haloperidol suppress cancer cell proliferation and migration, induce endoplasmic reticulum stress-mediated apoptosis, and reduce tumor growth and metastasis in mice (Jandaghi et al., 2016). On this basis, DRD2 antagonists have been proposed for clinical evaluation in pancreatic oncology (Bakadlag et al., 2019; Jandaghi et al., 2016).

Histamine H1 Receptor

Histamine is best known as an allergic mediator and central neurotransmitter, yet its H1 receptor (HRH1) has recently surfaced as a candidate therapeutic target in PDAC. A comprehensive bioinformatic screen of G-protein-coupled receptors ranked *HRH1* among the most intensely upregulated receptor genes in PDAC, displaying an average 20-fold elevation over normal pancreas (Insel et al., 2026). Salmerón and colleagues reported that HRH1 localizes predominantly to cancer cells across human and murine tumors, signaling via Gq/11 to elevate intracellular calcium and promote cell motility and invasion; oral treatment with the H1-antihistamine fexofenadine attenuated pancreatic tumor weight and downregulated immunomodulatory and fibrotic gene expression in genetically engineered mice (Salmerón et al., 2025). The same authors observed that retrospective epidemiological analyses linked H1-antihistamine prescriptions to improved overall survival in PDAC patients (Salmerón et al., 2025).

HRH1 signaling also impacts antitumor immunity. In PDAC cells, *HRH1* expression correlates inversely with HLA class I transcript levels and CD8+ T-cell infiltration, and HRH1 blockade restores surface MHC class I expression to enhance anti-PD-1 checkpoint therapy in orthotopic models (Zhong et al., 2024). A reciprocal positive feedback circuit between HRH1 and the transcriptional co-activator YAP1 was identified: HRH1 stabilizes and activates YAP1, whereas YAP1 promotes *HRH1* transcription, with elevated *HRH1* expression correlating with poor prognosis in public cohorts (Chen & Wen, 2026). These mechanisms are pertinent to CNS-active drugs, as several compounds, including benztropine, exhibit potent off-target H1-antagonist affinity alongside their primary neurological targets.

Repurposing CNS-Acting Drugs for Pancreatic Cancer

Drug repurposing offers an accelerated path to clinical testing in PDAC by bypassing early-phase safety and dosing trials. CNS-acting agents are well suited for cancer neuroscience because their receptor profiles overlap with the neurotransmitter pathways mediating neuro-epithelial communication. Table 1 summarizes the drug classes investigated to date and their corresponding levels of preclinical and clinical evidence.

Table 1. Selected CNS-acting and neurotransmitter-targeting drugs with evidence relevant to pancreatic cancer

Drug class / example	Main target(s)	Key evidence	Level of evidence
Non-selective β-blockers (e.g., propranolol, carvedilol)	ADRB1/ ADRB2	ADRB2–NGF feed-forward loop in mice (Renz, Takahashi, et al., 2018); improved immunotherapy in a stress model (W. Li et al., 2026); lower cancer-specific mortality with non-selective agents in cohort studies and meta-analysis (R. Li et al., 2026; Støer et al., 2021)	Preclinical + observational clinical

Muscarinic agonists (e.g., bethanechol)	CHRM1	Suppressed tumorigenesis and extended survival in mice (Renz, Tanaka, et al., 2018; expression of concern 2026); earlier stage at diagnosis in users (Cham et al., 2026)	Preclinical + observational clinical
Antipsychotic DRD2 antagonists (e.g., haloperidol, pimozide)	DRD2	Reduced proliferation, migration, xenograft growth and metastasis (Jandaghi et al., 2016)	Preclinical
NK-1R antagonists (e.g., aprepitant)	TACR1 (NK-1R)	Reduced neural invasion in 3D co-culture; synergy with paclitaxel (Schiedlauske et al., 2026)	Preclinical (in vitro)
H1-antihistamines (e.g., fexofenadine)	HRH1	Reduced tumor weight in mice; increased MHC-I and improved anti-PD-1 response (Salmerón et al., 2025; Zhong et al., 2024)	Preclinical (+ epidemiological signal)
Benztropine	CHRM1, HRH1, SLC6A3 (DAT)	Inhibition of breast cancer stem cells (Cui et al., 2017); suppression of tumor growth and metastasis via DAT/STAT3 (Sogawa et al., 2020); reduced tumor growth and oxaliplatin neuropathy in mice (Cerles et al., 2019)	Preclinical (no PDAC-specific studies)

Source: Compiled by the authors from the cited studies.

Among these candidates, β-blockers possess the largest clinical dataset, although survival associations are retrospective and restricted to non-selective agents (R. Li et al., 2026; Støer et al., 2021). DRD2 antagonists and NK-1R blockers demonstrate robust preclinical efficacy (Jandaghi et al., 2016; Schiedlauske et al., 2026). Evidence supporting H1-antihistamines spans molecular signaling, in vivo transgenic models, and synergistic immuno-oncology combinations (Salmerón et al., 2025; Zhong et al., 2024).

Benztropine, an anticholinergic approved for Parkinson's disease, illustrates both the opportunities and pitfalls of psychotropic repurposing. In a phenotypic screen of more than 2,500 approved drugs and drug-like molecules, benztropine mesylate selectively inhibited breast cancer stem cells, with functional assays implicating muscarinic, dopaminergic, and histaminergic receptors (Cui et al., 2017). In tumoroid models, benztropine reduced cellular viability and dampened STAT3, NF-κB, and β-catenin transcriptional activity, an effect attributed to the dopamine transporter (*SLC6A3*) (Sogawa et al., 2020). In mice, benztropine inhibited tumor growth, synergized with oxaliplatin, and prevented platinum-induced peripheral neuropathy (Cerles et al., 2019)—a clinically relevant finding given the routine use of oxaliplatin in FOLFIRINOX. Combination screens confirmed benztropine monotherapy activity against colorectal carcinoma lines and synergistic cytotoxicity when paired with paclitaxel in breast cancer models (Duarte et al., 2021, 2022).

Translating these preclinical findings to PDAC requires addressing three pharmacological considerations. Published evaluations of benztropine in pancreatic cancer models are currently lacking. Quantitative bioactivity profiling in ChEMBL demonstrates that benztropine binds HRH1 (Ki ≈ 0.37 nM) with affinity comparable to its canonical target CHRM1 (Ki ≈ 0.13 nM), whereas its affinity for the dopamine transporter DAT (Ki ≈ 193 nM) is several hundred-fold lower (Zdrazil et al., 2024). If muscarinic M1 signaling actively suppresses pancreatic tumor progression (Renz, Tanaka, et al., 2018), CHRM1 antagonism could prove counterproductive. Determining which of

these candidate targets is functionally present in patient-derived PDAC specimens is an essential prerequisite for rational translation, leading us to examine public genomic resources.

Exploratory Analysis of Public Datasets

We conducted an exploratory computational analysis across public transcriptomic datasets, including The Cancer Genome Atlas (TCGA), the Genotype-Tissue Expression (GTEx) project, the Clinical Proteomic Tumor Analysis Consortium (CPTAC), the Cancer Cell Line Encyclopedia (CCLE), and the Gene Expression Omnibus (GEO). This analysis examined three questions: (i) whether candidate neuroreceptors—particularly benztropine's three putative targets—are expressed in human PDAC; (ii) in which cellular compartments they reside; and (iii) whether their expression correlates with perineural invasion, spatial nerve proximity, molecular subtype, or patient survival. Table 2 summarizes the cohort specifications.

Table 2. Public datasets used in the exploratory analysis

Dataset	Samples analyzed	Data type	Purpose
TCGA-PAAD (Cancer Genome Atlas Research Network, 2017)	178 primary tumors; ductal adenocarcinomas only for survival (n = 145) and GSEA (n = 147)	Bulk RNA-seq (UCSC Xena, Toil-recomputed)	Expression, survival, pathway analysis
GTEx (GTEx Consortium, 2020)	167 normal pancreas samples	Bulk RNA-seq (UCSC Xena, Toil-recomputed)	Normal tissue reference
CPTAC-PDAC (Cao et al., 2021)	140 tumors; 133 with perineural invasion status	Bulk RNA-seq + pathology annotations	Perineural invasion, validation of survival
CCLE (Ghandi et al., 2019)	49 pancreatic cancer and 35 fibroblast cell lines	RNA-seq of cell lines	Cancer cell-intrinsic expression
GSE278687 (Chen et al., 2025)	21 Visium sections from 16 PDAC patients	Spatial transcriptomics	Proximity to nerves, cellular compartments

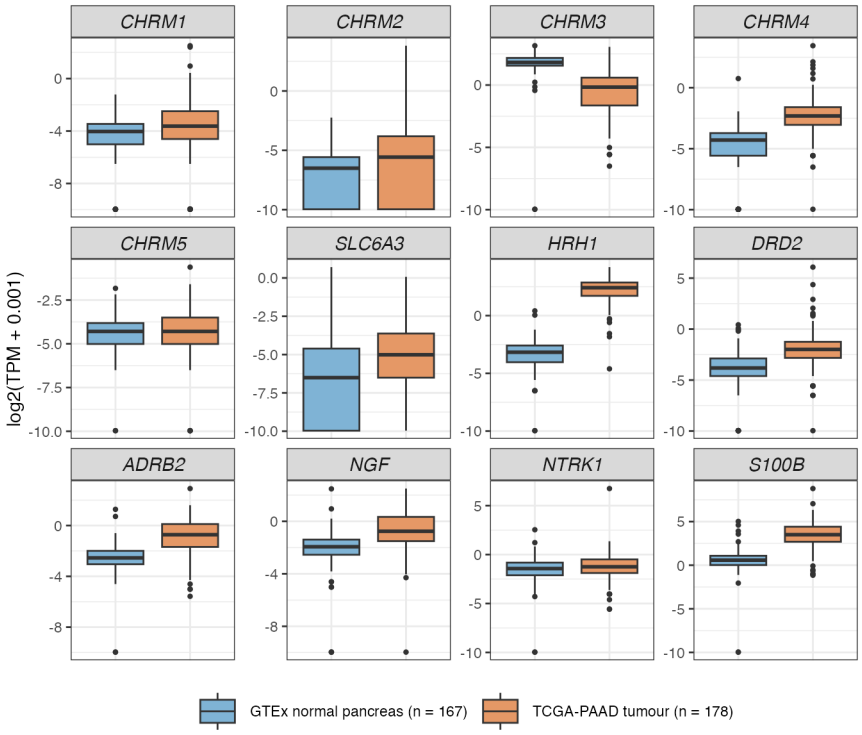
Source: Compiled by the authors.

Methods in brief. Expression data for primary TCGA-PAAD tumors and GTEx normal pancreas were accessed via the UCSC Xena Toil hub, in which both cohorts were processed through an identical RSEM-based workflow to enable direct comparison (Goldman et al., 2020; Vivian et al., 2017). Transcriptional profiles and clinical annotations for CPTAC-PDAC and CCLE were acquired through the cBioPortal application programming interface (Cerami et al., 2012; Gao et al., 2013). Survival analyses utilized curated overall and progression-free survival endpoints from TCGA (Liu et al., 2018), restricted to ductal adenocarcinoma histology. Differences between tumor and normal tissues were evaluated using the Wilcoxon rank-sum test and corrected for multiple testing using the Benjamini–Hochberg false discovery rate (FDR). Survival associations were modeled using Cox proportional hazards regressions on standardized expression values, adjusted for age and stage, and in sensitivity models, for acinar and islet cellular fractions alongside a continuous basal-minus-classical subtype score based on the gene signatures defined by Moffitt et al. (2015). Gene set enrichment analysis (GSEA) was performed on genes ranked by Spearman correlation with *HRH1*, using MSigDB Hallmark and KEGG pathways together with the classical and basal-like gene sets of Moffitt et al. (2015) (Kanehisa & Goto, 2000; Liberzon et al., 2015; Subramanian et al., 2005) and the fgsea algorithm (Korotkevich et al., 2016). Spatial transcriptomic Visium sections were analyzed

in Scanpy (Wolf et al., 2018); spots were classified as nerve, tumor, fibroblast, or immune spots based on marker gene scores, and the Euclidean distance from each tumor spot to the nearest nerve spot was calculated. To avoid treating thousands of spots within one section as independent observations, tumor spots adjacent to nerves (≤100 µm) and distant from nerves (>500 µm) were compared at the tissue section level using paired Wilcoxon tests. Analytical scripts are available from the corresponding author upon request.

Benztropine's classical targets are barely expressed in PDAC, whereas HRH1 is strongly upregulated. Among the 42 neurotransmitter-related genes examined, *CHRM1* was detected above 1 transcript per million (TPM) in only 3.4% of TCGA tumors, and *SLC6A3* in only 0.6% (Figure 2). In contrast, *HRH1* was expressed above this threshold in 96.6% of tumors but in only 1.2% of normal pancreas samples, corresponding to an approximately 48-fold higher median expression (log2 fold change 5.6). The muscarinic M3 receptor *CHRM3*, which is abundant in acinar cells, was lower in tumors, most likely reflecting the loss of acinar tissue rather than regulation within cancer cells. Markers of nerves and Schwann cells (*S100B*, *TUBB3*, *PRPH*) were higher in tumors than in normal pancreas, in line with reactive neural remodeling, and the NMDA receptor subunit *GRIN2D*, relevant to glutamatergic signaling, was among the most strongly upregulated genes (log2 fold change 5.6).

Figure 2. Expression of selected neurotransmitter receptor and nerve marker genes in TCGA-PAAD primary tumors (n = 178) compared with GTEx normal pancreas (n = 167).

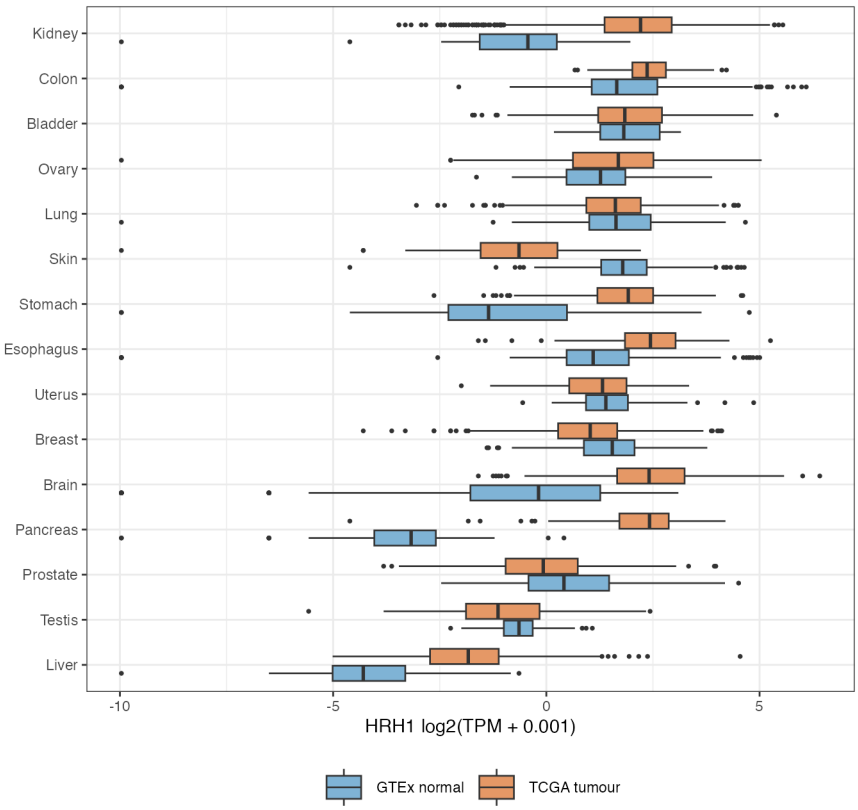


Values are log2(TPM + 0.001).

Source: Generated by the authors from TCGA and GTEx data obtained via the UCSC Xena Toil hub.

Across 15 tissues for which both TCGA tumors and GTEx normal samples were available, the increase in *HRH1* expression in tumors compared with matched normal tissue was largest in the pancreas, although *HRH1* was also increased in stomach, kidney, brain, and liver tumors (Figure 3). The large fold change in the pancreas partly reflects the very low *HRH1* baseline expression of normal pancreatic tissue. In cell lines, which lack stroma, all 49 pancreatic cancer cell lines expressed *HRH1* (median 3.7 reads per kilobase per million [RPKM]), whereas *CHRM1* remained below 1 RPKM in every pancreatic line. Fibroblast lines expressed *HRH1* at a similar level (median 4.2 RPKM), indicating that both cancer cells and fibroblasts are potential sources of *HRH1* in bulk tumor tissue.

Figure 3. HRH1 expression in TCGA tumors compared with matched GTEx normal tissues across 15 organs.



Source: Generated by the authors from TCGA and GTEx data obtained via the UCSC Xena Toil hub.

Bulk perineural invasion status leaves no detectable transcriptional signature. In CPTAC-PDAC, we compared tumors with (n = 116) and without (n = 17) perineural invasion. As a positive control, we first tested a nerve score composed of six nerve and Schwann cell markers, which would be expected to be higher in tumors with perineural invasion. This score did not differ between the groups ($p = 0.14$), and none of the 42 genes reached FDR < 0.05, either in unadjusted analyses or after adjustment for stromal fraction and neoplastic cellularity. The failure of the positive control indicates that bulk RNA-seq is unsuited to this question: perineural invasion is a focal microscopic finding recorded for the whole resection specimen, whereas RNA is extracted from a separate tissue fragment that may not contain an invaded nerve. The small size of the group without perineural invasion further limits statistical power.

HRH1 is associated with poorer survival, but this association is explained by the basal-like subtype. In age- and stage-adjusted Cox models, higher *HRH1* expression was associated with shorter overall survival in both TCGA (hazard ratio [HR] per standard deviation 1.44, 95% confidence interval [CI] 1.05–1.98) and CPTAC (HR 1.33, 95% CI 1.03–1.73), whereas higher *CHRM1* and *DRD2* expression was associated with longer survival in both cohorts (Table 3, Figure 4). However, *HRH1* expression correlated positively with a basal-minus-classical subtype score (Spearman ρ = 0.32 in TCGA and 0.46 in CPTAC), and *CHRM1* correlated negatively with it ($\rho = -0.40$ and -0.36). After additional adjustment for this subtype score, the associations of *HRH1* and *CHRM1* with survival were no longer significant in either cohort, and the association of *DRD2* remained significant only in TCGA (Table 3). Adjustment for acinar and islet content did not change the results. Because the basal-like subtype of PDAC is associated with worse outcome (Collisson et al., 2011; Moffitt et al., 2015), these results indicate that *HRH1* is a marker of the basal-like state rather than an independent prognostic factor. Separately, a higher nerve score was associated with longer survival

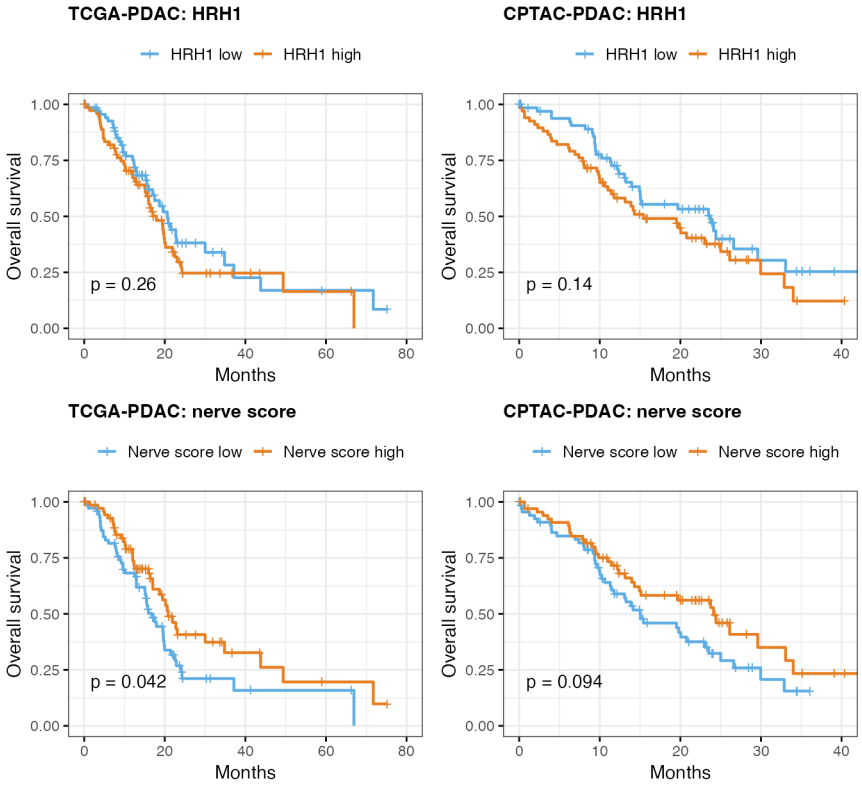
in CPTAC (HR 0.65, 95% CI 0.47–0.90) but not significantly in TCGA (HR 0.74, 95% CI 0.53–1.02); because this score was inversely related to both the classical and basal-like tumor programs and to proliferation gene sets in GSEA, it is likely to reflect lower tumor cellularity rather than nerve biology itself.

Table 3. Association of selected genes with overall survival in two PDAC cohorts (Cox proportional hazards models; hazard ratio per standard deviation of expression)

Gene	TCGA, age + stage HR (95% CI)	TCGA, + subtype score HR (95% CI)	CPTAC, age + stage HR (95% CI)	CPTAC, + subtype score HR (95% CI)
HRH1	1.44 (1.05–1.98) *	1.28 (0.91–1.79)	1.33 (1.03–1.73) *	1.12 (0.82–1.54)
CHRM1	0.72 (0.58–0.91) *	0.82 (0.63–1.05)	0.70 (0.54–0.90) *	0.81 (0.60–1.10)
DRD2	0.70 (0.53–0.92) *	0.70 (0.53–0.92) *	0.74 (0.57–0.96) *	0.84 (0.65–1.09)
NGF	0.76 (0.59–0.97) *	0.75 (0.60–0.94) *	0.81 (0.62–1.05)	0.93 (0.72–1.21)

*Source: Calculated by the authors from TCGA-PAAD and CPTAC-PDAC data. Note: * $p < 0.05$. TCGA: n = 145 (84 deaths); CPTAC: n = 131 (74 deaths). The subtype score is the mean of basal-like minus the mean of classical genes (Moffitt et al., 2015); models with the subtype score were also adjusted for acinar and islet content.*

Figure 4. Kaplan–Meier curves of overall survival according to HRH1 expression and nerve score in TCGA-PDAC and CPTAC-PDAC.



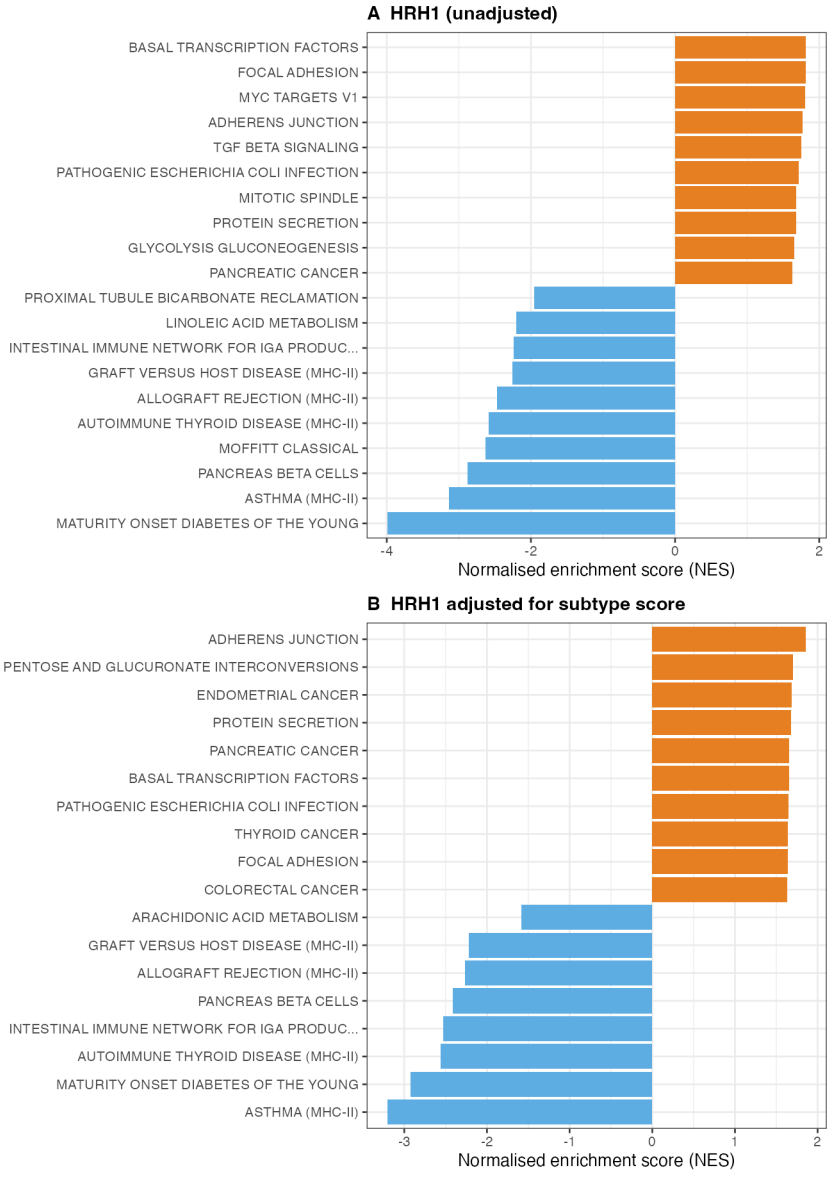
Patients were divided at the median; P values are from log-rank tests of this split, whereas the hazard ratios in Table 3 are from Cox models using continuous expression and are therefore not directly comparable.

Source: Generated by the authors from TCGA-PAAD and CPTAC-PDAC data.

HRH1-high tumors show a subtype-independent signature of TGF-β signaling, adhesion, axon guidance and low MHC class II expression. GSEA of 147 ductal adenocarcinomas showed that genes positively correlated with *HRH1* were enriched for epithelial–mesenchymal transition, hypoxia, TGF-β signaling, MYC and E2F targets, focal adhesion, and axon guidance, whereas genes of the classical subtype program were strongly depleted (normalized enrichment score [NES] −2.63,

FDR < 0.001) (Figure 5). When the analysis was repeated after removing the influence of the subtype score, the enrichment of epithelial–mesenchymal transition disappeared, but TGF-β signaling (NES 1.63, FDR = 0.002), proliferation-related gene sets, focal adhesion, and axon guidance (NES 1.49, FDR = 0.001) remained enriched. In both analyses, the most strongly depleted gene sets included islet-related programs (pancreatic β-cell and maturity-onset diabetes of the young gene sets), which most likely reflect less residual islet tissue in *HRH1*-high samples, and several gene sets driven by MHC class II genes (*HLA-DR*, *HLA-DQ*, and *HLA-DP*); the latter is consistent with the reported link between HRH1 and reduced antigen presentation (Zhong et al., 2024), although in bulk tissue this pattern may also reflect lower infiltration by antigen-presenting immune cells.

Figure 5. Gene set enrichment analysis of TCGA-PDAC tumors ranked by correlation with HRH1, (A) unadjusted and (B) after removing the effect of the basal-minus-classical subtype score.

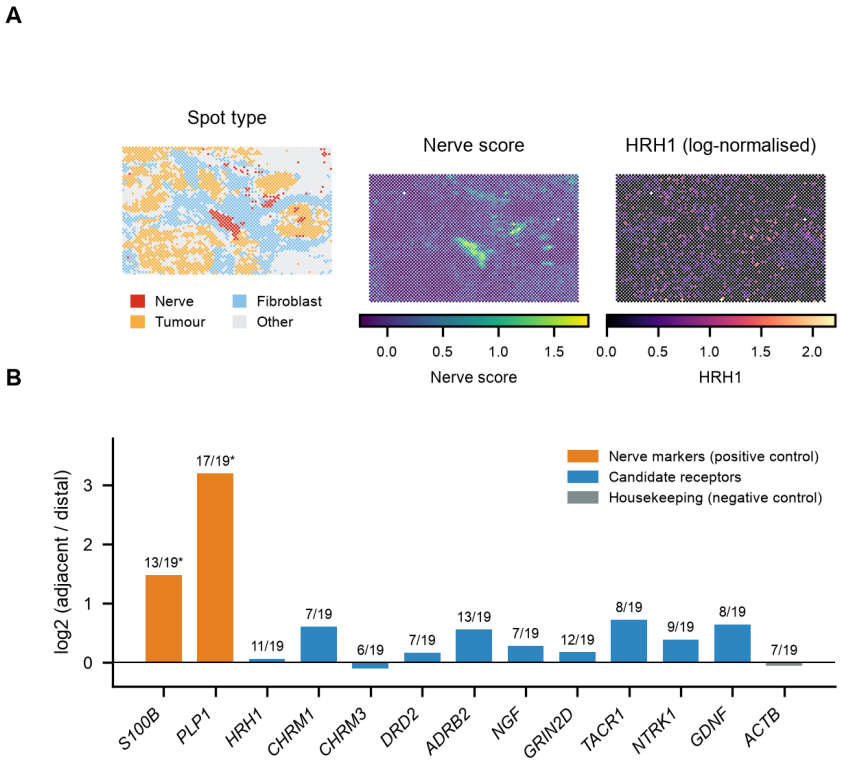


Bars show the ten most positively and negatively enriched gene sets (FDR < 0.05).
Source: Generated by the authors from TCGA-PAAD data using MSigDB gene sets.

Proximity to nerves does not change the expression of candidate receptors in tumor cells. Spatial transcriptomic data from PDAC sections with high or low neural invasion (Chen et al., 2025) were used to ask whether tumor regions adjacent to nerves express higher levels of candidate receptors. Nineteen of the 21 sections contained enough nerve and tumor spots for analysis. As

expected, the Schwann cell marker *PLP1* was higher in tumor spots adjacent to nerves than in distant tumor spots in 17 of 19 sections (paired $p < 0.001$), confirming that the approach detects nerve proximity (Figure 6). In contrast, *HRH1* was higher in adjacent spots in only 11 of 19 sections ($p = 0.68$), and none of the other candidate genes (*CHRM1*, *CHRM3*, *DRD2*, *ADRB2*, *NGF*, *GRIN2D*, *TACR1*, *NTRK1*, and *GDNF*) showed a consistent nerve-proximal increase. *HRH1* was detected at similar levels in tumor, fibroblast, immune, and nerve spots, in agreement with the cell line data.

Figure 6. Spatial transcriptomic analysis of PDAC sections.



(A) Representative section showing spot classification, nerve score and HRH1 expression. (B) Ratio of expression in tumor spots adjacent to nerves (≤100 µm) versus distant from nerves (>500 µm), calculated per section; PLP1 and S100B serve as positive controls and ACTB as a negative control.

Source: Generated by the authors from GEO dataset GSE278687 (Chen et al., 2025).

Summary of the analysis. The classical targets of benztropine, *CHRM1* and the dopamine transporter *SLC6A3*, are barely expressed in human PDAC tumors, and *CHRM1* is essentially absent from pancreatic cancer cell lines, whereas *HRH1*, which benztropine also binds with high affinity, is strongly and consistently upregulated. *HRH1* expression is linked to the aggressive basal-like state and to TGF-β, adhesion, and axon guidance programs, but not to physical proximity to nerves.

Challenges and Future Perspectives

Several challenges must be addressed before CNS-acting drugs can be repurposed for PDAC. The first concerns target expression. Many neurotransmitter receptors are expressed at low levels, and in bulk tissue their transcripts may originate from nerves, immune cells, or residual normal pancreas rather than from cancer cells. Our analysis illustrates this issue: *CHRM1*, the receptor through which muscarinic signaling has been proposed to suppress PDAC, was barely detectable in tumors or cell lines, and its apparent association with survival was explained by the classical subtype. Conversely, our finding that *HRH1* is expressed by both cancer cells and fibroblasts differs from the report that it is primarily a cancer cell receptor (Salmerón et al., 2025), a discrepancy that may reflect differences between transcript- and protein-level measurements, which should be resolved with immunohistochemistry or single-cell approaches.

The second challenge is polypharmacology. Most CNS drugs bind several receptors with comparable affinity, so the mechanism observed in cancer models may differ from the drug's neurological mechanism. For benztropine, available binding data make H1-receptor blockade at least as plausible a mechanism as muscarinic or dopaminergic effects. Future studies could test this directly by comparing benztropine with selective H1-antihistamines and by examining whether *HRH1* knockdown abolishes its effects in PDAC cells.

The third challenge is the strength of evidence. Most clinical data come from retrospective observational cohorts, which are vulnerable to confounding by indication, and some key preclinical findings are under editorial scrutiny. The molecular subtype of PDAC appears to be an important confounder: our analysis indicates that the prognostic value of *HRH1* largely reflects the basal-like state. Basal-like tumors have also been linked to heightened neural invasion potential (Chen et al., 2025), and the association of *HRH1* with TGF-β signaling is notable given that TGF-β induces the Schwann cell subset found at the leading edge of neural invasion (Chen et al., 2025). Whether HRH1 contributes to neural invasion in basal-like tumors, or is merely a marker of this aggressive state, remains an open question.

Finally, our analysis has limitations. It is exploratory and based on transcript levels; bulk perineural invasion annotations did not capture nerve-associated biology; the spatial data were limited to 21 sections; and none of the associations demonstrate causality. Nevertheless, integrating several independent data types, each with appropriate positive and negative controls, provides a useful framework for prioritizing targets before experimental work. Future studies combining spatial transcriptomics, protein-level validation, and functional experiments in patient-derived organoids or co-culture models of perineural invasion will be needed to determine which neurotransmitter pathways are truly actionable in PDAC.

Conclusion

The nervous system is an active component of the pancreatic cancer microenvironment. Perineural invasion, neural remodeling, and signaling through cholinergic, adrenergic, sensory, glutamatergic, dopaminergic, and histaminergic pathways all contribute to tumor progression, and drugs targeting these pathways are already approved for other indications. Among them, β-blockers have the most clinical data, while DRD2 antagonists, NK-1R antagonists, and H1-antihistamines have promising preclinical support. Our exploratory analysis of public datasets indicates that, for benztropine and related drugs, the histamine H1 receptor is a more plausible target in human PDAC than the muscarinic M1 receptor or the dopamine transporter. *HRH1* is strongly upregulated in PDAC, expressed by both cancer cells and fibroblasts, and linked to the aggressive basal-like state rather than to proximity to nerves. These findings support the further evaluation of H1-receptor blockade, and of CNS drugs with H1-antagonist activity, as a repurposing strategy in pancreatic cancer, ideally in molecularly defined patient subgroups.

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CHAPTER 2

EPITHELIAL–MESENCHYMAL TRANSITION AND CELL DEATH PLASTICITY IN CANCER: IMPLICATIONS FOR THERAPEUTIC RESISTANCE

1. Sabire GÜLER[1]

Introduction

Therapeutic resistance remains one of the major challenges in cancer treatment, limiting the durability of responses to conventional chemotherapy, molecularly targeted agents, and other anticancer interventions. Although the emergence of resistance has traditionally been associated with genetic alterations that reactivate oncogenic signaling or activate alternative survival pathways, accumulating evidence indicates that cancer cells can also adapt to therapeutic pressure through non-genetic mechanisms. These adaptations involve changes in transcriptional regulation, chromatin organization, cellular metabolism, and differentiation state, enabling subsets of malignant cells to survive treatment without necessarily acquiring new resistance-conferring mutations. Importantly, such phenotypic adaptations may initially be reversible but can facilitate

[1] Assoc. Prof. Dr., Bursa Uludag University, Faculty of Veterinary Medicine, Orcid: 0000-0002-7367-6859

the subsequent development of more persistent resistance (Sharma et al., 2010; Shaffer et al., 2017).

Among the cellular programs associated with tumor adaptation, epithelial–mesenchymal transition (EMT) has emerged as an important regulator of malignant progression and therapeutic response. EMT is a complex biological process in which epithelial cells undergo coordinated changes in cellular identity, adhesion, polarity, and cytoskeletal organization while acquiring features associated with mesenchymal differentiation. These changes are regulated by interconnected transcriptional networks and signaling pathways, including transforming growth factor-β (TGF-β) signaling and EMT-associated transcription factors such as SNAIL, TWIST, and ZEB family members. In cancer, activation of these programs can contribute to invasion, metastatic dissemination, cellular adaptability, and resistance to certain therapeutic interventions (Thiery et al., 2009; Nieto et al., 2016; Dongre & Weinberg, 2019). However, EMT should not be interpreted as a uniform or irreversible conversion between two distinct cellular phenotypes. Cancer cells may occupy a continuum of epithelial, hybrid epithelial–mesenchymal, and mesenchymal states, each characterized by different molecular and functional properties. Experimental investigations have identified multiple intermediate EMT states that differ in cellular plasticity, invasiveness, and metastatic potential, demonstrating that epithelial–mesenchymal heterogeneity represents a biologically relevant feature of tumor populations (Pastushenko et al., 2018). Furthermore, the expression of mesenchymal markers does not necessarily indicate the activation of a specific resistance mechanism. The biological consequences of EMT depend on tumor lineage, genetic background, microenvironmental conditions, and the signaling networks operating within individual cellular populations (Dongre & Weinberg, 2019).

An important consequence of epithelial–mesenchymal plasticity is its potential influence on the mechanisms regulating cancer cell survival and death. The effectiveness of many anticancer therapies depends on their ability to activate apoptosis following cellular damage or inhibition of essential oncogenic pathways. However, alterations in apoptotic signaling may allow malignant cells to survive despite adequate suppression of their therapeutic targets. In EGFR-mutant non-small cell lung cancer, for example, Song et al. (2018) demonstrated that EMT-associated ZEB1 suppresses the expression of the pro-apoptotic protein BIM, thereby reducing the apoptotic response to EGFR-targeted treatment. This finding illustrates how phenotypic reprogramming can influence therapeutic sensitivity through direct modulation of the cell death machinery. Nevertheless, resistance to apoptosis does not necessarily confer protection against other forms of regulated cell death. Ferroptosis, an iron-dependent process driven by the accumulation of oxidized membrane phospholipids, is mechanistically distinct from apoptosis and is regulated by cellular lipid metabolism and antioxidant defense systems (Dixon et al., 2012; Jiang et al., 2021). Experimental studies have demonstrated that certain cancer cell populations exhibiting mesenchymal characteristics or drug-tolerant states develop an increased dependence on glutathione peroxidase 4 (GPX4), a critical enzyme involved in the detoxification of lipid hydroperoxides. Disruption of this protective mechanism can selectively induce ferroptotic death in susceptible cancer cells (Viswanathan et al., 2017; Hangauer et al., 2017). These observations reveal an important biological paradox: cellular adaptations that reduce sensitivity to conventional anticancer therapies may simultaneously create vulnerabilities to alternative cell death mechanisms. However, the relationship between EMT and ferroptosis is not universal, and the acquisition of mesenchymal characteristics alone is insufficient to predict ferroptotic susceptibility. Rather, treatment responses are determined

by the interaction of cellular phenotype with specific apoptotic regulators, lipid metabolic pathways, and antioxidant dependencies. Understanding these relationships is essential for distinguishing phenotypic associations from causal resistance mechanisms and for identifying therapeutic strategies that exploit the molecular vulnerabilities of treatment-resistant cancer cells.

Molecular Regulation of EMT and Cellular Plasticity

Epithelial–mesenchymal transition (EMT) is controlled by a complex regulatory network that integrates extracellular signaling, transcriptional activity, epigenetic regulation, and cellular responses to environmental stress. Rather than being initiated by a single molecular event, EMT develops through coordinated changes in gene expression and cellular organization that influence adhesion, polarity, cytoskeletal dynamics, and interactions with the surrounding extracellular matrix. The intensity and duration of these changes vary considerably among tumor types, allowing cancer cells to acquire different degrees of mesenchymal differentiation while retaining aspects of their original epithelial identity (Lamouille et al., 2014; Nieto et al., 2016).

Transforming growth factor-β (TGF-β) is among the most extensively characterized extracellular regulators of EMT. Following ligand binding, TGF-β receptor complexes phosphorylate SMAD2 and SMAD3, which subsequently associate with SMAD4 and regulate transcriptional responses in the nucleus. These responses involve the activation of EMT-associated transcriptional regulators and changes in the expression of genes controlling epithelial differentiation and mesenchymal behavior. In addition to canonical SMAD-dependent signaling, TGF-β can engage noncanonical pathways, including MAPK and PI3K/AKT signaling, which modify cellular responses related to migration, survival, and cytoskeletal reorganization. Importantly, the biological

consequences of TGF-β activation depend on cellular context. Although TGF-β can inhibit proliferation in normal epithelial cells and certain early-stage tumors, alterations in oncogenic signaling and tumor-associated regulatory networks may shift its activity toward the promotion of invasion, cellular plasticity, and therapeutic resistance (Lamouille et al., 2014; Wang et al., 2023).

The transcriptional regulation of EMT is primarily associated with several interconnected families of transcription factors, including SNAIL (SNAI1), SLUG (SNAI2), TWIST1, ZEB1, and ZEB2. These regulators influence epithelial and mesenchymal gene expression through both direct transcriptional repression and broader changes in regulatory networks. Suppression of epithelial junction-associated genes, particularly CDH1, which encodes E-cadherin, can weaken intercellular adhesion and facilitate the reorganization of epithelial architecture. Concurrently, changes in the expression of cytoskeletal proteins, extracellular matrix components, and adhesion-associated molecules contribute to the acquisition of mesenchymal characteristics. However, these transcription factors do not operate as functionally equivalent regulators. Their effects depend on the cellular lineage, interacting cofactors, oncogenic background, and additional signaling pathways active within the tumor (Thiery et al., 2009; Lamouille et al., 2014; Nieto et al., 2016).

EMT-associated transcriptional programs are further regulated by interactions between transcription factors and epigenetic mechanisms. Changes in chromatin accessibility, histone modifications, and regulatory noncoding RNAs can influence the stability and reversibility of epithelial and mesenchymal characteristics. Reciprocal regulatory interactions between EMT-associated transcription factors and microRNAs, including the miR-200 family, contribute to the maintenance or modification of cellular identity. These regulatory interactions provide a molecular basis for the persistence of particular phenotypic states while also permitting

transitions in response to changes in extracellular signaling or environmental conditions (Lamouille et al., 2014; Nieto et al., 2016). An important consequence of this multilayered regulation is that EMT does not necessarily result in a complete or irreversible conversion to a mesenchymal phenotype. Cancer cells may retain epithelial characteristics while simultaneously expressing mesenchymal-associated molecules, generating intermediate or hybrid epithelial–mesenchymal states. These states are not merely transitional morphological appearances but can represent biologically distinct cellular populations with different functional properties. In experimental skin and mammary tumor models, Pastushenko et al. (2018) identified multiple populations distributed along the epithelial–mesenchymal spectrum and demonstrated differences in their plasticity, invasiveness, and metastatic potential. Their findings also revealed distinct transcriptional and epigenetic characteristics among these populations, supporting the concept that EMT is regulated through heterogeneous and context-dependent molecular programs. The existence of intermediate EMT states has important implications for the interpretation of experimental findings. Classification based exclusively on individual epithelial or mesenchymal markers may overlook the coexistence of multiple phenotypic characteristics within the same cell or tumor population. For example, reduced E-cadherin expression or increased vimentin expression may indicate changes associated with EMT but cannot independently establish the extent of cellular reprogramming or the functional consequences of that state. A more comprehensive assessment requires the integration of multiple molecular markers with phenotypic and functional analyses. Moreover, because epithelial–mesenchymal states can change over time, observations obtained at a single experimental endpoint may not adequately reflect the dynamic nature of cellular plasticity (Brabletz et al., 2018; Pastushenko et al., 2018). The molecular regulators of EMT can also influence biological processes beyond cellular adhesion and motility.

In particular, EMT-associated transcription factors may interact with survival pathways and modify the response to anticancer treatment. Experimental evidence in EGFR-mutant non-small cell lung cancer has demonstrated that TWIST1 contributes to resistance against EGFR-targeted therapies. Yochum et al. (2019) reported that targeting TWIST1-associated mechanisms enhanced sensitivity to EGFR inhibition in selected experimental models. These findings indicate that the functional importance of an EMT-associated transcription factor cannot be determined solely by its ability to induce morphological changes. Instead, its contribution to therapeutic resistance must be evaluated through the specific molecular and cellular processes that it regulates. EMT-associated signaling also intersects with pathways involved in cellular metabolism, stress adaptation, and survival. PI3K/AKT signaling is particularly relevant because it regulates multiple processes that can influence the survival of cancer cells under unfavorable conditions. Interactions between PI3K/AKT and EMT-inducing pathways may facilitate adaptive responses to extracellular signals and therapeutic stress. Nevertheless, PI3K/AKT activation occurs in many cancer cell states and is not specific to EMT. Therefore, its presence alone does not demonstrate that epithelial–mesenchymal reprogramming is responsible for a resistant phenotype. Establishing a causal relationship requires experimental evidence showing that modulation of the relevant pathway or EMT-associated regulator directly alters treatment sensitivity (Lamouille et al., 2014; Wang et al., 2023).

The relationship between EMT and therapeutic resistance is further illustrated by mechanisms involving the cellular response to DNA damage. In an experimental model of squamous cell carcinoma, Debaugnies et al. (2023) demonstrated that RHOJ, a small GTPase preferentially expressed in EMT-associated tumor cells, contributes to chemotherapy resistance by enhancing the

response to replication stress and facilitating the repair of treatment-induced DNA lesions. This mechanism differs from the direct inhibition of apoptotic signaling and demonstrates that EMT-associated resistance may arise through improved cellular tolerance of therapeutic damage. Importantly, these findings identify a specific functional mediator of resistance rather than attributing the resistant phenotype to mesenchymal differentiation alone.

The molecular complexity of EMT highlights its role as a dynamic and heterogeneous process that extends beyond changes in cellular morphology. EMT-associated regulatory networks influence cellular differentiation, adaptability, stress responses, and therapeutic sensitivity through distinct downstream mechanisms. However, the acquisition of mesenchymal characteristics does not necessarily confer resistance to anticancer treatment, as therapeutic outcomes depend on the specific molecular dependencies established within individual cellular states. Identifying the mechanisms that directly regulate cancer cell survival, particularly those affecting apoptotic susceptibility and alternative cell death pathways, is therefore essential for understanding the contribution of epithelial–mesenchymal plasticity to therapeutic resistance and for developing more effective therapeutic strategies.

EMT-Associated Mechanisms of Apoptotic Resistance

Apoptosis is a fundamental mechanism through which damaged, dysfunctional, or therapeutically targeted cancer cells are eliminated. The effectiveness of many anticancer agents depends not only on their capacity to disrupt oncogenic signaling or induce cellular damage but also on the ability of malignant cells to initiate and complete the apoptotic process. Consequently, alterations in apoptotic regulation can allow cancer cells to survive despite an apparently effective molecular response to treatment. Epithelial–mesenchymal transition (EMT) has been associated with such

alterations, although the mechanisms linking mesenchymal characteristics to apoptotic resistance differ among cancer types and therapeutic contexts (Song et al., 2018; Yochum et al., 2019).

The intrinsic apoptotic pathway is primarily regulated at the mitochondrial level by interactions among members of the BCL-2 protein family. Pro-apoptotic effector proteins BAX and BAK promote mitochondrial outer membrane permeabilization, whereas anti-apoptotic proteins, including BCL-2, BCL-XL, and MCL-1, restrict this process. BH3-only proteins, such as BIM, PUMA, and NOXA, respond to cellular stress and influence the balance between pro-survival and pro-death signals. Once mitochondrial outer membrane permeabilization occurs, the release of cytochrome c facilitates apoptosome formation and the subsequent activation of caspases responsible for cellular dismantling. The functional balance among these regulatory proteins determines the threshold required for apoptotic commitment and therefore influences the response to anticancer treatment (Czabotar et al., 2014; Czabotar & Garcia-Saez, 2023).

Changes in mitochondrial apoptotic competence can be particularly important during treatment with molecularly targeted agents. Although inhibition of an oncogenic driver may suppress downstream proliferative and survival signaling, this response does not necessarily result in cancer cell elimination. Cells with insufficient pro-apoptotic signaling or persistent anti-apoptotic protection may remain viable despite effective inhibition of the therapeutic target. This distinction between target engagement and apoptotic execution provides an important framework for understanding resistance mechanisms that arise independently of renewed oncogenic pathway activation.

Experimental evidence has established a direct relationship between EMT-associated transcriptional regulation and impaired apoptosis in selected lung cancer models. Song et al. (2018) demonstrated that mesenchymal EGFR-mutant non-small cell lung cancer (NSCLC) cells exhibited reduced sensitivity to EGFR inhibitor-induced apoptosis despite substantial suppression of EGFR-dependent signaling. Mechanistic investigations identified ZEB1 as a transcriptional regulator of this response. By binding to the promoter of BCL2L11, the gene encoding the pro-apoptotic protein BIM, ZEB1 suppressed BIM transcription and reduced the capacity of cancer cells to initiate apoptosis following EGFR inhibition. Genetic depletion of ZEB1 restored BIM expression, while pharmacological treatment with the BH3 mimetic navitoclax (ABT-263) enhanced apoptotic responsiveness in the experimental models examined. These findings are particularly relevant because they identify a molecular mechanism connecting an EMT-associated transcription factor to the apoptotic machinery. Rather than attributing resistance to mesenchymal differentiation itself, the study demonstrates how a specific transcriptional alteration can influence treatment-induced cell death. Reduced BIM availability can increase the threshold for mitochondrial apoptosis, allowing cancer cells to survive even when the targeted oncogenic pathway remains inhibited. This mechanism also suggests that assessment of apoptotic competence may provide information beyond conventional measurements of target inhibition or mesenchymal marker expression.

A related mechanism has been described for TWIST1 in EGFR-mutant NSCLC. Yochum et al. (2019) demonstrated that TWIST1 overexpression promoted resistance to EGFR tyrosine kinase inhibitors, including osimertinib, in experimental models. TWIST1 contributed to resistance partly through transcriptional suppression of BIM, whereas genetic or pharmacological

interference with TWIST1 increased treatment sensitivity. These findings indicate that distinct EMT-associated transcription factors can converge on components of the mitochondrial apoptotic pathway. Nevertheless, their relative contributions may vary according to the cellular background, and the presence of either transcription factor does not independently establish resistance in every tumor. The regulation of apoptotic susceptibility is also influenced by the balance between BH3-only proteins and anti-apoptotic BCL-2 family members. Pharmacological agents known as BH3 mimetics can interfere with selected anti-apoptotic proteins, thereby facilitating activation of the mitochondrial death pathway. In experimental settings where resistant cancer cells retain a functional apoptotic apparatus but exhibit an elevated apoptotic threshold, these agents may enhance sensitivity to targeted treatment. However, their effects depend on the specific anti-apoptotic proteins required for cellular survival, and therapeutic benefit cannot be predicted solely from the presence of EMT-associated characteristics (Czabotar et al., 2014; Song et al., 2018).

Another aspect of apoptotic resistance relevant to epithelial–mesenchymal plasticity is the ability of cancer cells to survive following detachment from the extracellular matrix. Under physiological conditions, epithelial cells depend on appropriate interactions with their surrounding matrix to maintain cellular organization and survival. Disruption of these interactions can activate anoikis, a form of apoptosis that limits the persistence of cells outside their normal tissue environment. During malignant progression, the acquisition of anoikis resistance may facilitate survival during local invasion and dissemination, thereby contributing to metastatic potential.

The functional importance of anoikis resistance was demonstrated by Douma et al. (2004), who identified the neurotrophic receptor TrkB as a mediator of anchorage-independent

survival and metastatic behavior in experimental systems. These findings established that suppression of detachment-induced apoptosis can promote malignant progression. However, anoikis resistance should not be considered an inevitable consequence of EMT. Although EMT-associated alterations in cellular adhesion and extracellular matrix interactions may facilitate detachment, continued survival requires additional molecular adaptations capable of preventing the activation of apoptotic pathways.

The relationship between EMT and treatment-induced cell death is further complicated by mechanisms that act upstream of apoptotic execution. Cancer cells may survive therapeutic exposure not only because their apoptotic machinery is less responsive but also because they more effectively tolerate or repair treatment-induced damage. For example, Debaugnies et al. (2023) demonstrated that RHOJ contributes to chemotherapy resistance in EMT-associated squamous cell carcinoma populations by promoting responses to replication stress and facilitating DNA damage repair. Although this mechanism can ultimately reduce treatment-induced cell death, it is mechanistically distinct from direct transcriptional suppression of pro-apoptotic proteins. These differences have important implications for the interpretation of therapeutic resistance. Reduced apoptosis following treatment may result from insufficient cellular damage, enhanced damage repair, impaired activation of pro-apoptotic signaling, or increased protection against mitochondrial permeabilization. Accordingly, the detection of a mesenchymal phenotype or reduced apoptotic activity is insufficient to identify the underlying resistance mechanism. Functional analyses of apoptotic regulators, mitochondrial responsiveness, and treatment-induced cellular damage are necessary to establish how EMT-associated molecular programs influence cancer cell survival.

The relationship between epithelial–mesenchymal plasticity and apoptotic resistance is therefore best understood through the specific survival mechanisms operating within individual cellular states. Identifying these mechanisms may support therapeutic approaches designed to restore apoptotic susceptibility in resistant cancer populations. At the same time, the persistence of cells with impaired apoptotic responses raises an important question regarding their sensitivity to other forms of regulated cell death, particularly those governed by lipid metabolism and oxidative membrane damage.

Ferroptosis Vulnerability in Mesenchymal Cancer Cell

Ferroptosis is an iron-dependent form of regulated cell death characterized by the excessive accumulation of oxidized phospholipids in cellular membranes. Unlike apoptosis, ferroptosis does not primarily rely on caspase activation or mitochondrial outer membrane permeabilization. Instead, its execution is determined by the balance between lipid peroxidation and the cellular mechanisms responsible for limiting oxidative membrane damage (Dixon et al., 2012; Jiang et al., 2021). A major protective mechanism against ferroptosis involves the cystine/glutamate antiporter system x^{c-}, whose transport activity depends on SLC7A11. Cystine uptake supports glutathione synthesis, enabling glutathione peroxidase 4 (GPX4) to reduce phospholipid hydroperoxides. Disruption of cystine availability, glutathione metabolism, or GPX4 activity can therefore promote ferroptotic death in susceptible cells (Yang et al., 2014). The transcription factor NRF2 also contributes to ferroptosis regulation by coordinating antioxidant and iron metabolism-related responses, although its effects depend on the cellular and metabolic context.

Ferroptotic susceptibility is additionally influenced by membrane lipid composition and alternative antioxidant systems. ACSL4 promotes the incorporation of polyunsaturated fatty acids into membrane phospholipids, increasing the availability of substrates for lipid peroxidation (Doll et al., 2017). In contrast, ferroptosis suppressor protein 1 (FSP1) provides a GPX4-independent protective mechanism, while dihydroorotate dehydrogenase (DHODH) contributes to mitochondrial ferroptosis defense (Doll et al., 2019; Mao et al., 2021). These findings demonstrate that ferroptosis sensitivity reflects multiple interacting metabolic and antioxidant pathways rather than dependence on a single molecular regulator.

The relationship between ferroptosis and epithelial–mesenchymal plasticity has particular relevance to therapeutic resistance. Viswanathan et al. (2017) identified a high-mesenchymal, therapy-resistant cancer cell state characterized by increased dependence on GPX4-mediated lipid peroxide detoxification. Similarly, Hangauer et al. (2017) demonstrated that drug-tolerant persister cells derived from different cancer models acquired vulnerability to GPX4 inhibition. Importantly, GPX4 disruption induced ferroptotic elimination of susceptible persister populations and prevented tumor relapse in a preclinical model. These observations suggest that cellular adaptations associated with resistance to conventional therapies may generate alternative metabolic vulnerabilities. Nevertheless, neither mesenchymal marker expression nor drug-tolerant status alone is sufficient to predict ferroptosis sensitivity. Differences in phospholipid composition, iron availability, antioxidant capacity, and cellular state may substantially influence treatment responses. Functional evaluation of lipid peroxidation and ferroptosis-specific rescue experiments is therefore necessary to establish whether a resistant

cancer cell population possesses a therapeutically exploitable ferroptotic vulnerability.

EMT, Cell Death Plasticity, and Therapeutic Resistance

Therapeutic resistance reflects the ability of cancer cells to maintain survival under treatment-induced selective pressure through genetic alterations, adaptive signaling, and changes in cellular identity. Epithelial–mesenchymal plasticity contributes to this process by modifying the cellular response to oncogenic pathway inhibition and cytotoxic stress. However, the relationship between EMT and resistance is highly dependent on tumor type, molecular background, and therapeutic context. EGFR-mutant non-small cell lung cancer (NSCLC) provides an important example of this complexity. Although EGFR tyrosine kinase inhibitors effectively suppress oncogenic signaling in sensitive tumors, cancer cells may persist through alterations in downstream apoptotic regulation or activation of alternative survival mechanisms. EMT-associated repression of BIM by ZEB1 illustrates how impaired apoptotic signaling can limit treatment-induced cell death despite inhibition of EGFR activity (Song et al., 2018). Resistance to osimertinib is nevertheless heterogeneous, involving EGFR-dependent alterations, bypass signaling, and phenotypic adaptations. EMT should therefore be considered one possible component of resistance rather than a universal explanation for therapeutic failure (Le et al., 2018).

Non-genetic drug tolerance introduces an additional dimension to therapeutic resistance. Small populations of cancer cells may survive initial treatment by entering adaptive states that do not necessarily involve stable resistance mutations. These drug-tolerant populations can subsequently contribute to disease recurrence or the emergence of more persistent resistance (Sharma et al., 2010; Shaffer et al., 2017). Importantly, adaptations that

support survival during one form of therapeutic stress may generate dependencies on alternative protective mechanisms. Increased reliance on GPX4-mediated lipid peroxide detoxification in selected mesenchymal and drug-tolerant populations provides an example of this phenomenon (Viswanathan et al., 2017; Hangauer et al., 2017).

The biological significance of EMT-associated resistance therefore lies not only in reduced sensitivity to conventional treatment but also in the distinct vulnerabilities that may accompany cellular adaptation. Identifying these vulnerabilities requires functional assessment of treatment responses rather than classification based exclusively on epithelial or mesenchymal markers.

Therapeutic Strategies and Emerging Opportunities

Understanding the molecular dependencies of resistant cellular states provides opportunities to develop therapeutic strategies that extend beyond inhibition of oncogenic signaling. One approach involves restoring apoptotic susceptibility in cancer cells that remain viable despite effective target inhibition. In experimental EGFR-mutant NSCLC models, pharmacological interference with anti-apoptotic BCL-2 family proteins enhanced the response to EGFR-targeted treatment, supporting the potential value of combining targeted therapies with agents that lower the apoptotic threshold (Song et al., 2018). However, differences in apoptotic protein dependency and treatment-related toxicity remain important limitations.

Targeting specific regulators of EMT-associated resistance represents another potential strategy. Experimental findings involving TWIST1 and RHOJ indicate that transcriptional regulation, cellular stress responses, and DNA damage tolerance can contribute to resistance through distinct molecular mechanisms (Yochum et al., 2019; Debaugnies et al., 2023). These observations

favor the identification of functionally relevant resistance mediators over indiscriminate suppression of EMT-associated signaling. Such selectivity is particularly important for pathways including TGF-β, whose biological activities vary across physiological and malignant contexts (Wang et al., 2023). Ferroptosis-based approaches offer a complementary possibility for targeting cancer cells with reduced apoptotic susceptibility. The identification of GPX4 dependency in selected therapy-resistant and drug-tolerant populations suggests that disruption of lipid peroxide detoxification may eliminate cells that survive conventional treatment (Viswanathan et al., 2017; Hangauer et al., 2017). Nevertheless, these findings remain predominantly preclinical, and the development of clinically applicable ferroptosis-inducing therapies is limited by pharmacological challenges, tumor heterogeneity, and potential effects on normal tissues.

Sequential treatment strategies may also be relevant when initial therapy generates residual populations with distinct metabolic dependencies. However, the effectiveness of such approaches must be established experimentally, including evaluation of treatment timing, potential antagonistic interactions, and toxicity. The identification of predictive biomarkers will similarly require integration of cellular phenotype with functional measurements of apoptotic competence and ferroptotic susceptibility rather than reliance on individual molecular markers.

The therapeutic implications of epithelial–mesenchymal plasticity extend beyond reversing mesenchymal differentiation. A more promising direction involves identifying the specific survival mechanisms and cell death vulnerabilities that emerge during treatment adaptation. Integrating molecular characterization with functional assessments of apoptosis and ferroptosis may help define more selective strategies for eliminating resistant cancer cell

populations, although their clinical applicability requires further validation.

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CHAPTER 3
DEEP LEARNING ARCHITECTURES AND TIME-LAPSE DYNAMICS IN IN VITRO FERTILIZATION: CURRENT STATUS, LIMITATIONS, AND FUTURE VISION

Running Title: (AI in IVF: Time-Lapse Dynamics)
Length of the manuscript in number of words: 3019

Abstract

Objective: Optimizing clinical pregnancy and live birth rates in assisted reproductive technologies depends largely on the selection, using noninvasive methods, of the embryo with the highest implantation potential. The interobserver variability of traditional morphological assessment methods has driven clinical embryology to develop more standardized, objective, and data-driven approaches.

Methods: This review examines the current applications of artificial intelligence architectures and time-lapse imaging systems (TLS) in reproductive medicine, the synergistic integration of these two technologies, and their impact on clinical decision-making processes, supported by the literature.

Results: Current evidence indicates that AI-assisted TLS offers significant advantages in terms of morphokinetic analyses, optimizing laboratory workflows, and predicting implantation success through deep learning algorithms. In addition, heterogeneity in training datasets, the lack of transparency in deep learning models (the “black box” phenomenon), the lack of external validation, and bioethical concerns are key barriers to implementation. Current research indicates that artificial intelligence should serve as an auxiliary tool to support the decisions of physicians and embryologists rather than replace them, and that progress in this field depends on large-scale, collaborative, data-driven clinical trials.

Keywords: Assisted Reproductive Technology, Artificial Intelligence, Time-Lapse Imaging, Embryo Selection, Cumulative Live Birth Rate

Introduction

Infertility, defined as the failure to achieve pregnancy within twelve months despite regular, unprotected sexual intercourse, is a multifaceted clinical syndrome that affects approximately 15% of couples of reproductive age worldwide (Zegers-Hochschild et al., 2009). In recent years, changes in lifestyle, the trend toward having children at an older age, environmental factors, and the rise in chronic diseases have contributed to a steady increase in the prevalence of infertility. For this reason, assisted reproductive technologies (ART) have emerged as one of the fastest-growing fields in modern reproductive medicine (Choudhary et al., 2025; Ye X. et al., 2025). Although in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) have undergone significant methodological evolution in recent years, live birth rates (LBR) per transfer remain plateaued at around 30–40 percent in the general population, depending on age (De Geyter et al., 2020; Ye C. et al., 2025). The primary physiological cause of this clinical impasse lies in the limitations of identifying genetically viable (euploid) embryos with high implantation potential

using non-invasive methods.

In traditional embryology practice, embryo selection is based on static morphological grading (e.g., the Gardner and Schoolcraft criteria) performed under a light microscope at specific developmental stages (Gardner & Schoolcraft, 1999; Jeong, 2025). However, this "snapshot" approach has two major methodological drawbacks. First, the fact that embryos are exposed to changes in temperature, pH, and osmolality during the process of transferring culture plates from the incubator to the microscope puts epigenetic programming and embryo viability at risk (Swain, 2014; Gatimel et al., 2026). Second, static morphological scoring is subjective in nature; studies show that Cohen's kappa coefficient for inter-observer and intra-observer assessments often remains at suboptimal levels (Storr et al., 2017). These limitations have accelerated the integration of the concept of "precision medicine" into reproductive medicine (AlSaad et al., 2025). With the introduction of time-lapse imaging systems (TLS) into routine laboratory practice, embryos can now be monitored continuously without being removed from optimal incubator conditions, and a new spatiotemporal dataset—referred to as "morphokinetics"—has been introduced into the literature (Campbell et al., 2025; Luong & Le, 2024; Meseguer et al., 2011). However, manually processing the massive volume of biological data (big data) generated by TLS exceeds cognitive limits. In this context, artificial intelligence—particularly machine learning (ML) and deep learning (DL) architectures—has emerged as a key catalyst for transforming spatiotemporal data into clinical insights (Fernandez et al., 2020; Saranya et al., 2026).

Applications of Artificial Intelligence in Reproductive Medicine

Deep learning architectures that go beyond the histological and morphological limitations of the embryology laboratory are being integrated into all diagnostic and therapeutic stages of infertility management. Regardless of TLS, artificial intelligence (AI) applications in clinical practice can be summarized as follows:

Advanced Sperm Analysis

Convolutional neural networks (CNNs) can detect cellular and subcellular abnormalities in high-magnification morphological examination (IMSI) images, overcoming the limitations of traditional computer-assisted sperm analysis (CASA) systems (AlSaad et al., 2025). Studies conducted by McCallum et al. have demonstrated the potential of artificial intelligence to non-invasively predict sperm head vacuolization and the DNA fragmentation index, thereby enabling the selection of the most optimal spermatozoa for microinjection in cases of severe male-factor infertility (McCallum et al., 2019).

Follicular Dynamics and Endometrial Receptivity

Artificial intelligence-powered image processing algorithms (e.g., U-Net architectures) standardize antral follicle counting by performing automatic segmentation on 2D/3D ultrasound images (Khosravi et al., 2019; Kothari et al., 2026). On the other hand, the integration of endometrial thickness data with transcriptomic profiles—such as the ERA (Endometrial Receptivity Array)—through machine learning provides models with high predictive value for determining a personalized "implantation window" (AlSaad et al., 2025; Blesa et al., 2014;

Dahiphale et al., 2024).

Clinical Decision Support Systems (CDSS)

Support Vector Machines and Random Forest algorithms integrate heterogeneous variables such as maternal age, BMI, AMH, and basal FSH to optimize gonadotropin dosing and minimize the risk of ovarian hyperstimulation syndrome. Additionally, these models enable the prediction of patient-specific cumulative live birth rates (Dehghan et al., 2025).

Time-Lapse Imaging (TLS) and Morphokinetic Analysis

The temporal kinetics of development provide much deeper insights into the embryo's genetic and metabolic competence than static morphology alone. TLS consists of high-resolution digital microscopy systems integrated into closed incubator setups, providing the continuous biological data required for AI algorithms.

Maintenance of Physiological Stability

Keeping embryos in a completely isolated system eliminates environmental stress factors. Multiple focal-plane images recorded at regular intervals (10–20 minutes) map the embryo's developmental journey at the cellular level.

Morphokinetic Markers

Meseguer et al. were the first to standardize the finding that the timing of successive cleavage events (t2, t3, t4, t8) and cell cycle intervals (cc2, s2) in embryos show a strong correlation with implantation success (Meseguer et al., 2011). Other studies also confirm that the ploidy rates of embryos divided within specific optimal morphokinetic time windows are significantly higher (Chavez et al., 2012).

Detection of Abnormal Cleavage Patterns

Anomalies such as direct cleavage, reverse cleavage, and blastomer multinucleation—which are often overlooked by conventional microscopy—can be clearly detected using TLS. These abnormal phenomena are directly associated with a high risk of aneuploidy and low viability (Zaninovic & Rosenwaks, 2020).

Artificial Intelligence and Time-Lapse Integration: Decision Automation

Artificial intelligence's computer vision algorithms transform TLS hardware from a passive incubator into an active clinical decision support system (CDSS) (Zaninovic & Rosenwaks, 2020).

Automatic Scoring (Auto-Annotation)

Deep learning models detect morphological events—such as the appearance/disappearance of pronuclei (tPNf) and the onset of cavitation (tM)—with millisecond precision without human intervention. Automatic annotation systems eliminate intra- and inter-observer variability and dramatically reduce the operational workload in the embryology laboratory (AlSaad et al., 2025).

Implantation Prediction and Hierarchical Ranking

AI-based commercial or experimental models (e.g., iDAScore, KIDScore AI) analyze the embryo's preimplantation development sequence on a pixel-by-pixel basis (Chavez-Badiola et al., 2020). Deep learning (DL) algorithms determine an implantation score specific to each embryo by evaluating cytoplasmic granulation dynamics, zona pellucida changes, and cellular asymmetries that the human eye cannot detect.

Clinical Benefits

The implications of the integrated AI-TLS ecosystem for clinical practice are multifaceted:

Standardization

Evaluation based on objective and mathematical criteria establishes quality standardization across laboratories.

Safe Transfer Policies

These systems, which can distinguish minimal differences between embryos of the same morphological quality (e.g., 4AA blastocysts), encourage clinics to implement "single-embryo transfer" policies, thereby reducing the incidence of multiple pregnancies—the most significant complication of ART.

Quality Management

Optimizing workflows allows laboratory directors to focus on quality control and quality assurance procedures.

Methodological Limitations and Barriers

Specific methodological barriers stand in the way of this technological revolution being accepted as the "gold standard" in clinical practice:

Lack of External Validation

Current deep learning architectures are typically trained using similar patient groups (homogeneous cohorts) and standard culture conditions at a single center. The fact that the system learns only within this narrow framework raises the possibility of a significant decline in its predictive performance when integrated into clinics with different patient demographics or different laboratory workflows (Bori et al., 2020).

The Black Box Problem

Medical ethics require that clinical decisions be justified. The fact that DL models cannot explain, with transparent causality, the specific parameters on which they base their high or low scores for an embryo gives rise to the need for "Explainable AI" (XAI) (Luong & Le, 2024).

Data Bias

An uneven distribution of demographic factors—such as age and genetic background—or of positive/negative clinical outcomes (prognostic data) in the datasets used to train AI algorithms directly affects the system's learning process. This imbalance can cause the model to develop a

bias against cellular characteristics or rare patient profiles (minority phenotypes) that are underrepresented in the dataset, leading it to operate with a higher margin of error when evaluating these groups (Luong & Le, 2024).

Bioethical and Legal Perspectives

In addition to technical limitations, ethical and legal considerations are also gaining increasing importance. The use of autonomous algorithms in reproductive medicine presents unique ethical and legal dilemmas. Issues such as the processing of large-scale patient data, data security, algorithmic biases, the transparency of AI decisions, and the sharing of clinical responsibility are at the center of not only technological but also ethical and regulatory debates (Boucret et al., 2025). The European Union's Artificial Intelligence Act (AI Act), the General Data Protection Regulation (GDPR), and national data protection regulations form the fundamental legal framework that will shape the future of applications in this field (Jeong, 2025). While cloud-based genetic analyses pose privacy and cybersecurity risks, the high setup costs of these systems limit access to this technology for patients with low socioeconomic status, thereby creating inequity in healthcare (Meggle, 2025; Rajendran et al., 2025). Furthermore, it remains unclear under current healthcare law regulations whether liability for medical malpractice arising from the discard of a healthy embryo as a result of a false-positive or false-negative algorithmic score lies with the software developer or the clinician.

Discussion and Conclusion

A current analysis of the literature demonstrates that the integration of time-lapse imaging and artificial intelligence signals an irreversible paradigm shift in histology and embryology laboratories. Although CNN-based models have become the standard approach in current studies, memory-based architectures such as RNNs and LSTMs play an indispensable role in analyzing not only the instantaneous morphology of the embryo but also its temporal developmental dynamics (AlSaad et al., 2025; Luong & Le, 2024). Indeed, in today's clinical practice, the highest success rates are achieved through the use of hybrid (ensemble) systems that combine image and temporal analysis, rather than relying on a single algorithm.

However, it should not be forgotten that technical successes do not always translate directly into clinical successes. Although retrospective cohort studies show that artificial intelligence models achieve high Area Under the Curve (AUC) values in embryo selection, meta-analyses in the Cochrane database remain cautious regarding whether this technological superiority translates into a universal and statistically significant increase in "live birth rates" (Armstrong et al., 2018). In the future, new-generation algorithms capable of processing massive datasets—such as the Vision Transformer—are expected to redefine embryo selection standards. However, for these advanced technologies to become the standard of care in clinical practice, it is essential to increase algorithmic transparency, create bias-free multinational databases, and validate the clinical efficacy of these systems through multicenter, prospective, randomized controlled trials (RCTs). The most fundamental way to reduce algorithmic bias is to diversify training datasets by integrating data from different geographic regions, clinical settings, and patient backgrounds

(including different ethnic origins, age groups, etc.) (AlSaad et al., 2025). By allowing clinics to train models together without directly exporting sensitive data, federated learning solves major privacy concerns. This secure approach brings highly diverse and inclusive demographics into the system, effectively reducing AI biases that stem from a lack of data (Luong & Le, 2024). The integration of AI systems into IVF laboratories is not merely a technological revolution; it is a complex process that requires forward-looking research based on multicenter, approved protocols, in which ethical boundaries, clinical responsibilities, and data protection laws are strictly monitored.

In conclusion, artificial intelligence should not be viewed as an autonomous system that replaces the cognitive and analytical processes of physicians and clinical embryologists, but rather as a powerful "second opinion" (copilot) tool that supports the decision-making process with objective data. The reproductive health ecosystem of the future will be built on a multidisciplinary foundation where biological expertise and algorithmic power are integrated within ethical and legal boundaries.

Abbreviations

AI	Artificial Intelligence
ML	Machine Learning
DL	Deep Learning
IMSI	Intracytoplasmic Morphologically Selected Sperm Injection
CNN	Convolutional Neural Network
BMI	Body Mass Index
AMH	Anti-Müllerian Hormone
FSH	Follicle Stimulating Hormone
IVF	In Vitro Fertilization
ICSI	Intracytoplasmic Sperm Injection
ART	Assisted Reproductive Technology
ERA	Endometrial Receptivity Array
TLS	Time-Lapse System
CDSS	Clinical Decision Support Systems

CASA	Computer Assisted Sperm Analysis
RNN	Recurrent Neural Network
LSTM	Long Short-Term Memory
AUC	Area Under the Curve
RCT	Randomized Controlled Trial

Disclosure Statement

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Kaynakça/References

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