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REVIEW ARTICLE

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Stromal barriers to nanomedicine penetration in the pancreatic tumor microenvironment

Hiroyoshi Y. Tanaka[1] | Mitsunobu R. Kano[1,2]

[1]Department of Pharmaceutical Biomedicine, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan

[2]Department of Pharmaceutical Biomedicine, Okayama University Graduate School of Interdisciplinary Science and Engineering in Health Systems, Okayama, Japan

Correspondence
Mitsunobu R. Kano, Department of Pharmaceutical Biomedicine, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan; Department of Pharmaceutical Biomedicine, Okayama University Graduate School of Interdisciplinary Science and Engineering in Health Systems, Okayama, Japan.
Email: mikano-tky@umin.net

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Pancreatic cancer is known for its dismal prognosis despite efforts to improve therapeutic outcome. Recently, cancer nanomedicine, application of nanotechnology to cancer diagnosis and treatment, has gained interest for treatment of pancreatic cancer. The enhanced permeability and retention (EPR) effect that promotes selective accumulation of nanometer-sized molecules within tumors is the theoretical rationale of treatment. However, it is clear that EPR may be insufficient in pancreatic cancer as a result of stromal barriers within the tumor microenvironment (TME). These limit intratumoral accumulation of macromolecules. The TME and stromal barriers inside it consist of various stromal cell types which interact both with each other and with tumor cells. We are only beginning to understand the complexities of the stromal barriers within the TME and its functional consequences for nanomedicine. Understanding the complex crosstalk between barrier stromal cells is challenging because of the difficulty of modeling pancreatic cancer TME. Here we provide an overview of stromal barriers within the TME. We also describe the preclinical models, both in vivo and in vitro, developed to study them. We furthermore discuss the critical gaps in our understanding, and how we might formulate a better strategy for using nanomedicine against pancreatic cancer.

KEYWORDS
drug delivery, nanomedicine, pancreatic cancer, stromal barrier, tumor microenvironment

1 | INTRODUCTION

Pancreatic cancer has a dismal prognosis despite intensive research over the last several decades.[1] A recent development in treatment is approval of albumin-bound nab-paclitaxel in combination with gemcitabine. This prolongs median survival from 6.7 months (for gemcitabine alone) to 8.5 months.[2] The basic assumption behind nab-paclitaxel is the EPR effect, first proposed in 1986.[3] The EPR hypothesis suggests that tumor neovasculature is immature with underdeveloped lymphatic drainage. This leads to increased leakage of macromolecules from blood vessels (enhanced permeability) and accumulation of leaked macromolecules (enhanced retention).

The EPR effect is the theoretical basis of cancer nanomedicine[4] and its application to diagnosis and/or treatment. However, nanomedicine has yet to reach its full potential. It is becoming increasingly clear that a major hurdle is the existence of a heterogeneous TME[5,6] in which there exists complex tumor-stromal crosstalk.[7,8] We therefore aim to provide

Abbreviations: ABC, accelerated blood clearance; CAST, cancer stroma targeting therapy; EPR, enhanced permeability and retention; GEMM, genetically engineered murine model; PDGF, platelet-derived growth factor; PDX, patient-derived xenograft; PSC, pancreatic stellate cell; TGF-β, transforming growth factor-β; TME, tumor microenvironment.

an overview of the TME and its importance to nanomedicine efficacy in pancreatic cancer. Importantly, we will refer to the biological and physical obstacles that the TME poses to nanomedicine penetration of the tumor as "stromal barriers". We also look at preclinical models of pancreatic cancer and stress the importance of developing clinically relevant models sufficiently recapitulating the characteristics of the TME to promote and accelerate studies on stromal barriers.

2 | STROMAL BARRIERS TO DRUG DELIVERY WITHIN THE TME IN PANCREATIC CANCER

The TME consists of various stromal cell types,[9] and these prevent penetration of nanotherapeutic agents into tumors, thus limiting efficacy.[10] Stromal barriers consist both of the cells and their secreted products. For this reason, it is informative to analyze the stromal tissue architecture of the cancer in question. Pancreatic cancer is characterized by fibrosis, and a nanotherapeutic agent, given i.v., must first extravasate and pass through a thick, fibrous tissue to locate a tumor target (Figure 1).

We have previously shown, in a murine BxPC-3 xenograft model of pancreatic cancer, that pharmacological inhibition of TGF-β signaling reduced pericyte coverage and increased intratumoral accumulation of nanotherapeutic agent. This improved efficacy[11,12]: extending the known role of pericytes in physiological vessel stabilization[13] to hindrance of nanoparticle extravasation. Furthermore, histopathological analyses of various human cancers showed that variable pericyte coverage correlated significantly with chemotherapeutic response. For example, we observed prominent pericyte coverage in pancreatic cancer in clear contrast to colon cancer. The latter cancer lacks pericyte coverage and is generally more responsive to chemotherapy.[14,15] We also found that while the CT26 colon carcinoma model with little pericyte coverage shows optimal intratumoral nanoparticle accumulation when treated with the angiogenesis inhibitor Sorafenib, the BxPC-3 model responds only to TGF-β inhibition.[16] This suggests that vessel architecture, notably in relation to pericyte coverage, is an important determinant of nanotherapeutic efficacy. Thus, understanding and exploiting abnormalities in vessel architecture may lead to better accumulation of nanotherapeutic agents in pancreatic cancers (Figure 2).

Furthermore, Smith et al have reported varying responses to anti-angiogenic agents in tumors relative to vascular and stromal architecture. They provide a conceptual framework to explain these variations,[17] and suggest that tumors can be divided into those with a tumor-vessel phenotype (blood vessels are distributed among cancer cells), and those with a stromal-vessel phenotype (blood vessels are embedded within stroma surrounding cancer cells). Only the first type is responsive to anti-angiogenic drugs. Pancreatic cancer, with its characteristic, desmoplastic morphology, is of the stromal-vessel phenotype,[18,19] and thus requires an alternative targeting strategy (such as TGF-β inhibition) to facilitate nanomedicine penetration of the tumor.

Furthermore, recent reports show that fibrotic stroma—consisting of PSC and secreted ECM components such as collagen and hyaluronan[20,21]—also constitute a barrier to drug delivery.[18,22-24] Fibrosis is thus considered a target in improving drug delivery in pancreatic cancer.[25] However, the way in which fibrotic elements

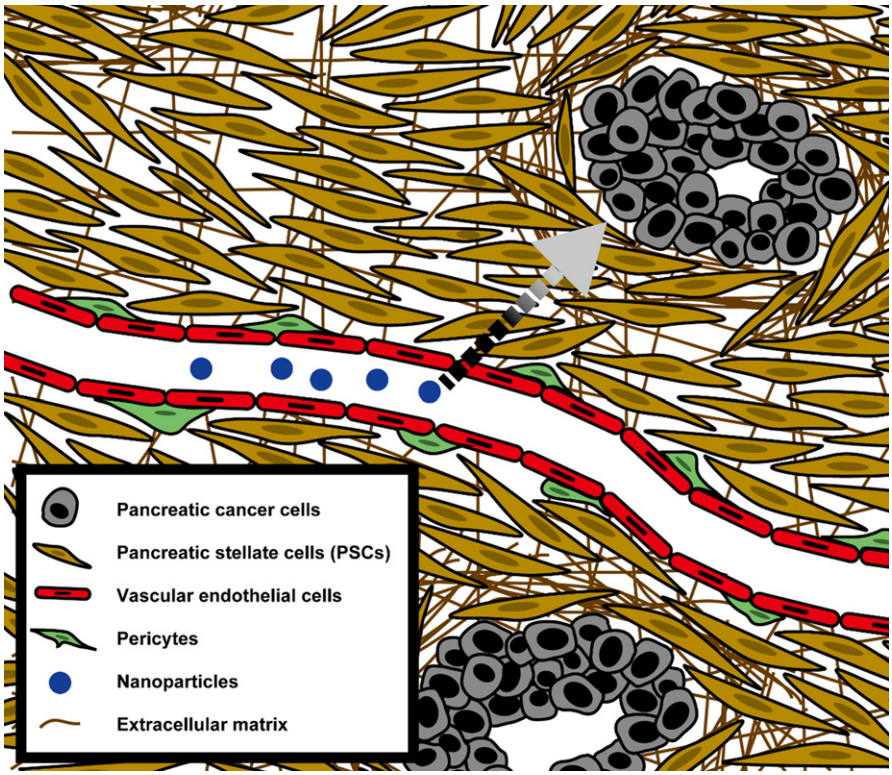

FIGURE 1 Pancreatic cancer microenvironment. The tumor microenvironment (TME) consists of numerous cell types and extracellular matrix, which collectively affect drug delivery. Pancreatic cancer is notably characterized by fibrosis separating cancer cells from blood vessels. The dotted arrow shows the path that an i.v. given nanoparticle must travel to reach cancer cells and achieve its effects

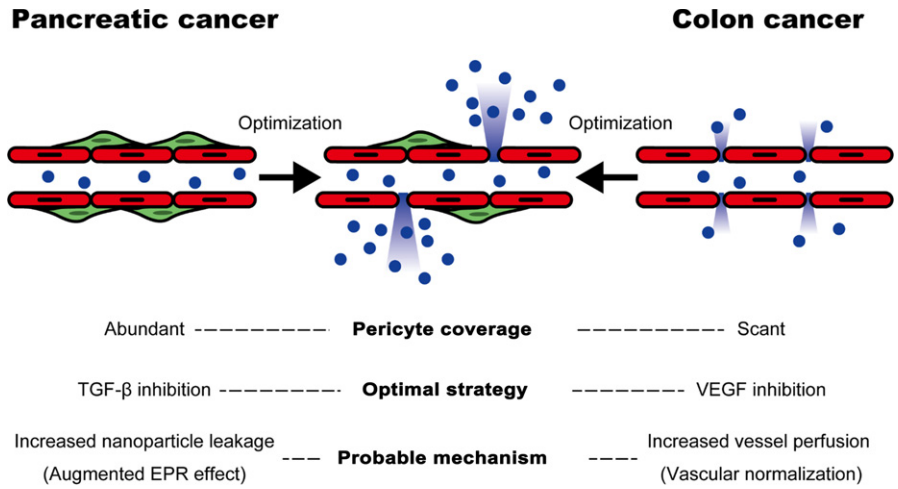

FIGURE 2 Pericyte coverage of intratumoral vessels affects vascular function and nanoparticle extravasation. Intratumoral vessels with varying levels of pericyte coverage show different profiles regarding nanoparticle extravasation: pancreatic cancer vessels have abundant pericyte coverage. Transforming growth factor beta (TGF-β) inhibition reduces pericyte coverage and results in increased nanoparticle leakage possibly as a result of augmented enhanced permeability and retention (EPR) effect. Colon cancer, characterized by vessels with little pericyte coverage, is shown for comparison. Unlike pancreatic cancer, the optimal strategy to increase nanoparticle extravasation is vascular endothelial growth factor (VEGF) inhibition. This increases perfusion by normalization of the vasculature

block drug delivery is unclear: it may involve physical obstruction as a result of increased ECM deposition,[24,26,27] decreased stromal vessel density,[18,28] and vessel compression and collapse [22,23] (Figure 3).

3 | PRECLINICAL IN VIVO MODELS OF PANCREATIC CANCER

The presence of stromal barriers within the TME of pancreatic cancer that limit therapeutic efficacy underscores the need for preclinical models that recapitulate the essential components of the TME. Here, and in the following section, we describe current models and the steps being taken towards development of new models. This topic has recently also been reviewed elsewhere.[29,30]

The current way of demonstrating efficacy of a particular formulation of nanomedicine is in vivo, usually with mice. Murine pancreatic cancer models can be generally divided into cell-line-based xenograft models, GEMM, and PDX models (Table 1). Cell-line-based xenograft models are generated by inoculation of pancreatic cancer cell lines, and have been widely used, mostly for their relative ease of use. Although xenografts, generated by inoculation of the BxPC-3 cell line, show prominent fibrosis—especially when given together with fibroblast growth factor-2 as we have reported[24]—most cell-line-based xenografts fail to show appreciable levels of fibrosis. In contrast, GEMM, that rely on recapitulating mutations observed in human pancreatic cancer in genes such as *Kras*, in combination with *Tp53*, *Cdkn2a*, *Smad4*, and *Tgfbr2*, more closely mirror the histopathology of human pancreatic cancer.[31-35] However, they usually take more time to fully develop and thus are more time-consuming than cell-line-based xenografts. Another method, now gaining momentum, is the PDX model. This engrafts tumor specimens from patients. PDX models of pancreatic cancer reportedly retain and/or recapitulate many features of human disease,[36-39] and have enabled large-scale screening in mice.[40] However, engraftment efficiency is not uniform and has been associated with adverse clinicopathological features in the patient of origin, which may confound results.[41]

4 | PRECLINICAL IN VITRO MODELS OF PANCREATIC CANCER

An exciting new development is introduction of in vitro models with 3D culture techniques such as spheroid culture,[42,43] organotypic/organoid culture,[44-47] and layer-by-layer ECM nanofilm coating-based culture[19,48] (Figure 4). The advantage, compared to conventional culture (ie, cells cultured 2D on plastic), is that it facilitates modeling of complex intercellular and/or cell-ECM interactions.

Spheroid culture is usually made with cell-repellent cultureware[43] and/or the hanging drop method.[42] In this way, 3D cultures of both pancreatic cancer cells and PSC have been generated, and used to analyze tumor-stromal interaction and nanoparticle penetration. Organoid cultures use ECM gels in which cell lines or cells/tissues obtained from surgery or biopsy are embedded. These have been used both for analyses in vitro, and transplanted into mice to model the histopathology of human disease.[44] We have used the layer-by-layer ECM nanofilm coating method, that sequentially builds a nanofilm of ECM components on the cell surface,[49] to construct models of the desmoplastic reaction in pancreatic cancer and assess nanoparticle penetration of fibrotic tissue.[19,48] The technique also allows generation of open-ended, vascular networks within 3D tissue, and may be used to analyze nanoparticle extravasation from the vasculature.[50,51]

For the present, in vitro 3D tissue studies cannot fully replace in vivo studies. However, they will complement in vivo studies when

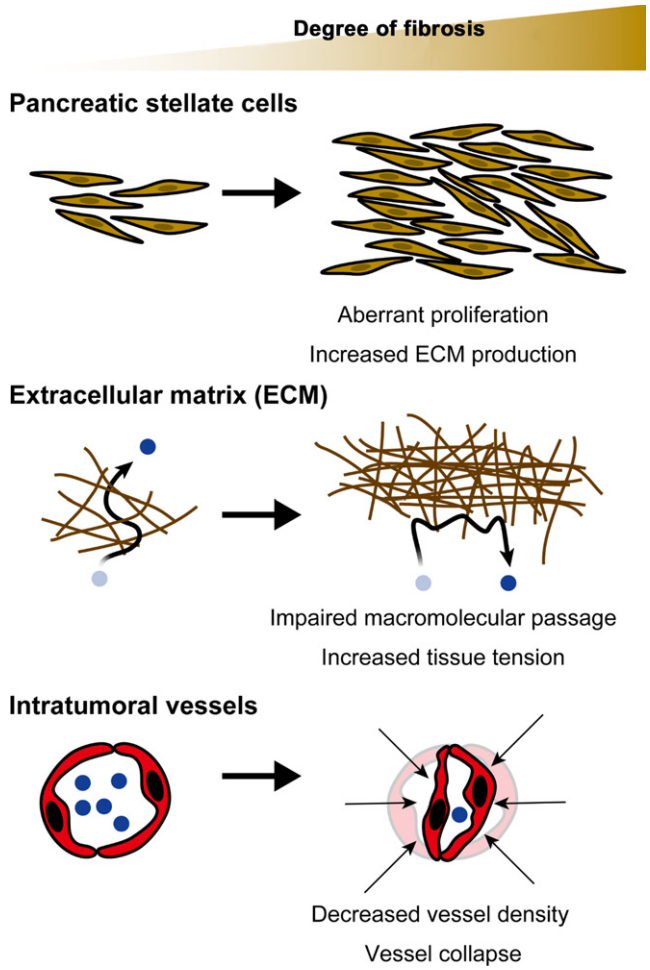

FIGURE 3 Effect of fibrosis on delivery of nanotherapeutics. Pancreatic cancer has prominent fibrosis. Pancreatic stellate cells are the main constituent of fibrosis in pancreatic cancer, and readily produce extracellular matrix (ECM) components such as collagen. The abundantly produced ECM is a physical barrier to macromolecules. It also increases tissue tension resulting in vascular compression and collapse, thus reducing intratumoral perfusion and nanotherapeutic delivery

molecular scale analysis is required and high spatiotemporal resolution may be cumbersome or challenging. They may also help interpret the complex crosstalk within the TME.

5 | GAPS IN OUR UNDERSTANDING OF STROMAL BARRIERS: FUTURE DIRECTIONS

Here, we discuss major gaps in our understanding of stromal barriers within TME, and examine how to fill these. Although the EPR effect has been a useful guide to nanotherapeutics, our understanding of nanoparticle extravasation is limited. For example, we know that extravasation and subsequent intratumoral accumulation depend on nanoparticle size,[12] but we do not know why. Furthermore, although the EPR effect suggests static pores within tumor neovessels, Stirland et al[52] report that nanoparticles of the same size, injected at different times, do not colocalize. We also know that dynamic bursts, or nano-eruptions, in tumor blood vessels lead to accumulation of nanoparticles within the tumor,[53] but we do not know how nano-eruptions occur. However, their existence does suggest that the static image of leaky tumor vessels conjured by the EPR hypothesis needs to be modified. Notably, with greater understanding of genotypic and phenotypic alterations in tumor endothelial cells,[54,55] the relationship between such alterations, and occurrence of nano-eruptions, is a highly interesting question. The 3D models detailed above[50,51] may help us better understand this complexity.

Another important question concerns the role of fibrotic stroma in pancreatic cancer and how this may be therapeutically overcome. A number of reports show that simple ablation of fibrotic cells within pancreatic cancer results in disease progression.[28,56,57] Thus, a more sophisticated strategy of reprogramming fibrotic cells into a tumor-suppressive phenotype may be required.[58,59] This is consistent with genomic and transcriptomic analyses reporting molecularly distinct subtypes of pancreatic cancer,[60-62] with distinct stromal expression signatures that serve as prognostic indices independent of tumor cells.[63] Furthermore, we have found that pancreatic cancer patients

TABLE 1 Frequently used animal models of pancreatic cancer

Animal model	Advantages	Drawbacks
Cell-line-based xenografts	Easy to perform, especially when s.c. inoculated Short experiment duration Comparison with in vitro results is intuitive	Often lacks stroma Immunological involvement is difficult to assess because of use of immunodeficient mice Cell lines may not faithfully represent cancer cell population found in human primary tumors
Genetically engineered mouse models (GEMM)	Recapitulates human histopathology well Well characterized and reproducible	Expensive Time-consuming
Patient-derived xenograft (PDX) models	Recapitulates human histopathology well Patient-specific mechanisms may be addressed	Engraftment efficiency is not high and may select for aggressive tumors Labor intensive Expensive Reproducibility may become a concern as a result of patient specificity

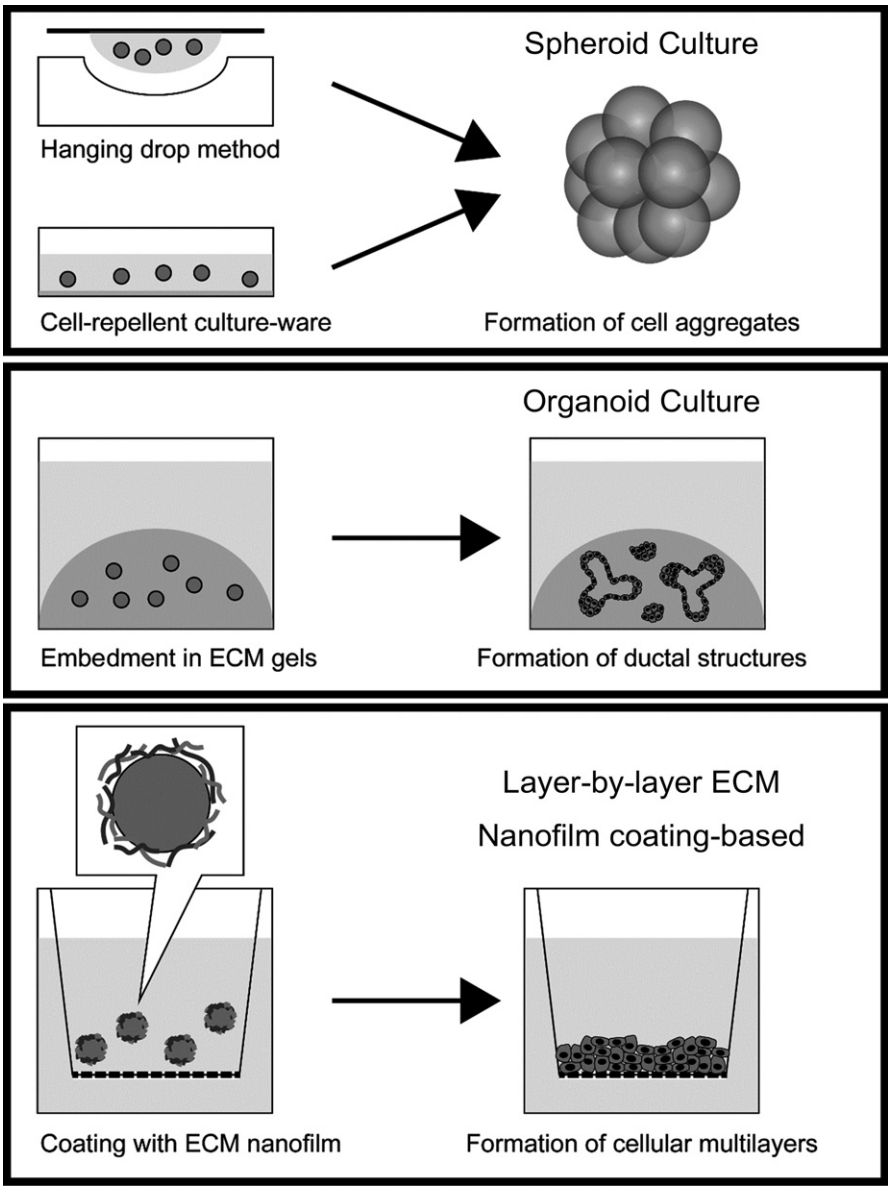

FIGURE 4 Various 3D culture methods. Spheroid culture is achieved through the hanging drop method or through use of cell-repellent culture-ware. Organoid culture is achieved by embedment of cells in extracellular matrix (ECM) gels. Layer-by-layer ECM nanofilm coating-based culture is achieved by creating an ECM nanofilm on the surface of cells prior to cell seeding. Multiple cell types may be mixed in these 3D culture methods to generate in vitro models of pancreatic cancer tumor microenvironment. For simplicity, only a single cell type is depicted in this figure

with high positivity for PDGF receptor-β in stroma have a worse prognosis.[64] Although studies assessing the importance of these distinct subtypes to nanomedicine have not yet been carried out, it may be a good approach, given increased expression of collagen proteins in those "activated" stromal subtypes with worse prognosis[63] and our knowledge of the role of PDGF signaling in fibrosis.[65] However, Laklai et al[66] have also emphasized the importance of tissue tension in pancreatic cancer prognosis. Experimental conditions with varying levels of fibrosis, which presumably demonstrate different tissue tension as well, must thus be compared with caution. Indeed, mechanical forces, such as interstitial fluid pressure and tissue tension, are not easily manipulated experimentally as independent variables and consequently remain largely under-studied.

The long-term safety and efficacy of nanomedicine is also an urgent issue. For example, nanotherapeutic formulations often use an outer coating of PEG to increase biocompatibility, but PEGylated nanoparticles are more rapidly cleared after repeated injections: a process known as ABC.[67,68] The ABC phenomenon is immunological —caused by generation of IgM antibodies—and may be clinically problematic in multiple dose treatment.[69] Furthermore, research on cobalt-chromium nanoparticles indicates possible DNA damage propagated across cellular barriers.[70,71] Therefore, immunogenicity and mutagenicity of nanoparticles, the mechanisms by which they develop, and their biological/clinical consequences for the patient, are all topics that require further study.

Finally, in addition to passive targeting through the EPR effect, the development of active targeting by specific markers within TME for intratumoral accumulation of nanotherapeutics also requires study.[72,73] A number of candidate targets for pancreatic cancer is under consideration and has been reviewed elsewhere.[74] Furthermore, uptake and intracellular behavior of nanoparticles by individual cells must also be tailored to prevent premature degradation and to facilitate optimal therapeutic effect (a concept known as "subcellular targeting").[75,76] However, as with passive targeting by EPR, stromal

barriers within TME will likely hinder passage of actively targeted nanotherapeutics. This is especially the case following antibody conjugation for active targeting, which further increases the size of nanotherapeutic formulation. Matsumura recently proposed the CAST strategy to circumvent this difficulty. Using antibodies, it first targets extracellular components, such as collagen IV, insoluble fibrin, and tissue factor, within stroma to make a scaffold from which conjugated low-molecular cytotoxic agents can be released and diffuse freely.[27]

6 | CONCLUSIONS

The existence of stromal barriers within TME which limit therapeutic efficacy of nanomedicine is now clearly established. However, how these barriers develop and how they hinder therapy is far from clear. Both increased knowledge of the complex crosstalk within TME, and preclinical models that accurately show the complexity of TME, in vivo and in vitro, are needed to advance research and treatment of pancreatic cancer.

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CONFLICT OF INTEREST

Authors declare no conflicts of interest for this article.

ORCID

Hiroyoshi Y. Tanaka http://orcid.org/0000-0003-1494-7716
Mitsunobu R. Kano http://orcid.org/0000-0003-1593-1855

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