Novel Therapeutics and Pre-clinical Imaging for Pancreatic Cancer – View from the Lab

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Abstract: Pancreatic cancer is a devastating disease with a five-year survival rate of 6%. A key driver of disease progression is the tumour microenvironment, which is characterised by fibrosis. A dynamic interplay between tumour cells, pro-fibrogenic pancreatic stellate cells and a dense extracellular matrix impedes effective drug delivery and promotes chemoresistance and metastases. In addition, mutations in pancreatic cancer are highly heterogeneous, making it difficult to effectively treat all patients with one approach. Thus, any effective pancreatic cancer treatment should consider targeting both pancreatic cancer and the stromal compartment. While basic research has provided promising new leads on therapeutic targets for this disease, many of them remain "undrugable" by conventional approaches. Advances in nanoparticle technology and intravital preclinical imaging of live tumours is providing new insight into the behaviour of the disease *in vivo* and guiding how best to target this disease with higher specificity and lower off-target toxicity. Here, we describe in brief, key advancements in both rapidly emerging fields and highlight their current and future application in the treatment of pancreatic cancer.

Pancreatic Cancer and the Tumour Microenvironment

Pancreatic cancer is the fourth leading cause of cancer-related deaths in Western nations, with a dismal five-year survival rate of 6% [1]. By 2030, pancreatic cancer is predicted to become the second leading cause of

cancer-related deaths in Western nations [2]. The poor prognosis of this disease is attributed to late clinical presentation, metastasis and chemoresistance. A major driver of the aggressive nature of this disease is the pancreatic cancer microenvironment [3].

Pancreatic cancer is characterised by extensive stromal reaction or fibrosis (scar tissue), which surrounds and dwarfs the tumour elements [4]. Fibrosis distorts the tumour vasculature, creating an oxygen-poor (hypoxic) and nutrient deprived environment [5, 6]. In addition, the fibrosis can act as a physical barrier to drug delivery [3]. These conditions are known to drive chemoresistance and metastatic spread in cancer cells. The stromal reaction is complex and involves multiple cell types, including immune, endothelial, fibroblasts and stellate cells [4]. Depending on the stage of disease and by virtue of their cellular function – stromal cells may either aid or inhibit tumour growth. However, this review will focus on pancreatic stellate cells (PSCs), which are the major pro-fibrogenic cell type in the stroma of pancreatic cancer [7]. In the healthy pancreas, PSCs play an important role in tissue repair and maintenance of extracellular matrix proteins. However, activation of PSCs by tumour cells causes them to rapidly proliferate and deposit excessive amounts of fibrotic proteins [7].

In addition, crosstalk between tumour cells and PSCs exists within pancreatic tumours. Cancer cells secrete growth factors to recruit PSCs, while activated PSCs reciprocate by secreting cytokines and fibrotic proteins regulating tumour cell proliferation, apoptosis, invasion, metastasis and chemoresistance (**Figure 1**) [7]. This complex interaction between tumour cells, PSCs and the surrounding microenvironment is a major reason many therapeutic approaches have failed in pancreatic cancer and needs to be considered when designing new therapeutic approaches for the disease.

Current Pancreatic Cancer Chemotherapies

Gemcitabine has long been the first line treatment for patients with unresectable pancreatic cancer. Recent years have seen a move to chemotherapeutic regimens using multiple drugs to try and overcome chemoresistance. Gemcitabine and abraxane (albumin-bound paclitaxel) combination therapy is now a current standard of care for unresectable pancreatic cancer [8]. More aggressive polychemotherapeutic regimens such as FOLFIRINOX (combination of folinic acid, 5-fluorouracil, irinotecan and oxaliplatin) are also being applied in the clinic [9]. While these new approaches have significantly improved patient survival, the survival benefit is only in the vicinity of a few extra months (Gemcitabine + Abraxane: extends median

survival by 8 weeks over gemcitabine alone; FOLFIRINOX: extends median survival by 17 weeks over gemcitabine alone) [8, 9]. Moreover, the high toxicity of regimens like FOLFIRINOX also restricts their application to the fittest patients. Clearly, new therapeutic approaches are needed to achieve major improvements in patient survival.

New Therapeutic Targets

As mentioned earlier, there are two main hurdles that make pancreatic cancer so hard to treat: the chemoresistant nature of the cancer cells and the extensive fibrosis orchestrated by pancreatic stellate cells. Any effective pancreatic cancer treatment should target *both* the pancreatic cancer cells and the stromal reaction (**Figure 1**). On this front, recent discoveries at the bench have identified novel therapeutic targets that hold promise for reprograming the stroma, modulating fibrosis dynamics and enhancing our ability to kill pancreatic tumour cells.

Targeting the Stromal Reaction

Several studies have demonstrated the potential benefit of targeting aspects of the stromal reaction in tissue culture and mouse models of pancreatic cancer. For example, the anti-fibrotic drug pirfenidone (used to treat idiopathic pulmonary fibrosis) has been applied in a clinically relevant mouse model of pancreatic cancer [10]. The group used an orthotopic pancreatic cancer mouse model (direct injection of pancreatic cancer cells and PSCs into the mouse pancreas) and demonstrated that oral administration of the drug reduced fibrosis, tumour growth and metastatic spread, and improved the efficacy of gemcitabine [10]. More recently, novel work has shown that the Vitamin D receptor on PSCs is a master regulator of their cancer-promoting phenotype [11]. The group used calcipotriol, a vitamin D analog (used in the treatment of psoriasis), and administered this into a clinically relevant spontaneous pancreatic cancer mouse model. This resulted in a significant reduction in fibrosis and improved drug access into pancreatic tumours, and when therapeutically combined with gemcitabine, a 57% increase in survival [11]. Studies by Provenzano et al [12] and Jacobetz et al [13] demonstrated that enzymatic depletion of hyaluronic acid (abundant component of pancreatic fibrosis) in a spontaneous pancreatic cancer mouse model, improved drug access and efficacy. More recently, a landmark paper by Miller and colleagues [14] highlighted how the texture of fibrosis can predict pancreatic cancer patient outcome. The group also showed that by inhibiting lysyl oxidase, an enzyme that cross-links collagen (thus controlling fibrosis texture), reduced the stiffness of fibrosis, thereby suppressing tumourigenesis and metastatic spread and enhancing gemcitabine efficacy [14]. These studies continue to

highlight the need to reprogram the stroma in order to overcome a major barrier to pancreatic cancer treatment. However, caution must be taken when targeting the stroma, as studies have demonstrated some components can help contain pancreatic cancer [15, 16], but this is very much dependent on the therapeutic gene target and what cells in the stroma are affected. Thus, it is important to examine the effects of targeting one stromal component on other cells in the stroma, in clinically relevant models.

Targeting Pancreatic Cancer Cells

The growing understanding of pancreatic cancer cell biology has allowed scientists to focus on the development of more cancer cell-specific molecular targets for pancreatic cancer. Major goals for researchers studying pancreatic cancer include to identify new molecular targets in pancreatic cancer that can impair tumour growth and metastasis, reduce off-target toxicity compared to systemic administration of drugs and radiation, and improve the efficacy of existing therapeutics. Targets fitting these criteria often come from unexpected cellular networks. For example, McCarroll et al [17] recently demonstrated the potency of βIII-tubulin as a therapeutic target for pancreatic cancer. This protein is a component of the cell cytoskeleton, the upregulation of which had been previously associated with drug resistance in a variety of cancers [18]. The group showed for the first time that βIII-tubulin was a critical survival factor in pancreatic cancer cells [17]. Inhibition of βIII-tubulin was able to halve tumour growth and metastatic spread in a clinically relevant orthotopic pancreatic cancer mouse model [17]. Ideal therapeutic targets for pancreatic cancer are not restricted to proteins. For example, microRNA-21, a small RNA sequence that downregulates tumour suppressors, is upregulated in pancreatic cancer [19, 20]. Inhibition of this target in a mouse model of pancreatic cancer was also able to reduce tumour growth and increase tumour sensitivity to gemcitabine [21]. Similarly, other studies have related microRNA profiling with clinicopathological criteria and pancreatic cancer patient outcome [22] highlighting the future promise of this technology. While targets like these hold great promise for pancreatic cancer treatment, their translation to the clinic may be hindered by their “undrugable” status, that is, there are currently no traditional small molecule inhibitors that can inhibit them. Exciting new progress in the field of nanotechnology is set to challenge this perception as recently demonstrated in other cancer types [23].

Nanoparticle Therapeutics: Targeting the ‘Undrugable’

Nanoparticles are delivery vehicles in the nanometre scale, ideally between 10-100 nanometres in diameter [24, 25]. They are capable of carrying a drug or RNA interference (RNAi) therapeutics. RNAi makes it

possible to inhibit any target of interest at the gene level, with high specificity. Nanoparticle technology is already in use in the clinic (for example albumin-bound paclitaxel, now used for the treatment of metastatic pancreatic cancer; **Table 1**) and in clinical trials for a variety of cancers (**Table 2**). More recently, Boyer et al [26] published a first-generation nanoparticle being developed for delivery to pancreatic tumours and demonstrated that this nanoparticle was capable of delivering and releasing RNAi therapeutics into pancreatic cancer cells *in vitro* [26].

While nanoparticles have been designed in a variety of shapes, charges and sizes, they all must be stable in the bloodstream and must be able to deliver their cargo to tumour cells if they are to be used in a therapeutic setting [27]. Nanoparticles are often charged to enable them to bind their cargo. However, this charge can trigger immune responses and bind proteins in the blood that hinder the function of the nanoparticle. One way scientists have improved the stability of nanoparticles is by the addition of neutral charged polymers, to the surface of nanoparticles. For example, the addition of polyethylene glycol (PEG) has been used to effectively mask nanoparticles from detection by immune cells, allowing increased time in the bloodstream [28]. An appealing feature of nanoparticles is the ability to target them to specific cell types by attaching targeting proteins or compounds to their surface. This reduces off-target toxicity commonly associated with conventional chemotherapy [29]. For example, studies have employed vitamin A-conjugated nanoparticles to deliver RNAi therapy to hepatic stellate cells and PSCs in mouse models of hepatic and pancreatic fibrosis, respectively [30, 31]. Both cell types display elevated levels of cell surface vitamin A receptor expression [32]. The group demonstrated that they could effectively deliver RNAi therapy to inhibit a protein involved in production of fibrosis, specifically in stellate cells [30]. Notably, these nanoparticles/RNAi therapies were able to resolve pancreatic and hepatic fibrosis [30, 31].

Nanoparticles therefore have the potential to transform treatment for pancreatic cancer, especially in the context of recent advances in pancreatic cancer genomics. We now know that there are only a few common mutations in pancreatic cancer, making personalised medicine essential [33]. Using nanoparticles/RNAi therapies and advanced genomics, clinicians could eventually be able to administer a specific cocktail of RNAi therapeutics based on the genetics of a patient's tumour, with minimal off target toxicity and high efficacy. In addition, nanoparticles can be applied in combination therapies, and to package and deliver enzymes or drugs (for example, abraxane; **Table 1**) to specific cell types, thus avoiding off-target toxicity and enhancing tumour penetrance.

Preclinical Imaging in Pancreatic Cancer

Various imaging technologies are used to monitor pancreatic cancer in the clinic, including computed tomography, transabdominal ultrasound, magnetic resonance imaging and endoscopic ultrasonography [34]. In parallel, high resolution preclinical imaging technologies from the laboratory setting are being employed to embrace the complexity of biological events that occur during the aetiology of the disease. These approaches have shed light on the spatiotemporal regulation of events driving pancreatic cancer at the single cell and subcellular levels. Here, we provide a brief overview of how complementary preclinical imaging approaches offer insight into the molecular bases of pancreatic cancer and can improve the development of new therapies in the disease [35].

Imaging the Tumour Microenvironment of Pancreatic Cancer

As previously discussed, pancreatic cancer progression occurs in a complex three-dimensional microenvironment with reciprocal feedback from the surrounding host tissue. *In vitro* models combined with immunohistochemistry analysis of patient tissues have been extensively employed to characterise the pancreatic tumour microenvironment [4, 36, 37]. While these approaches give insights into the interactions between cancer cells and their surrounding stroma, they are rather static and therefore do not fully recapitulate the contextual intricacy of pancreatic cancer biology. Direct imaging of stromal components however has revealed insights into the complexity of tissue structures and functions during disease development.

Second Harmonic Generation (SHG) imaging, a label free technique, has been used to characterise the extra-cellular matrix texture and organisation in relation to pancreatic cancer fibrosis. For instance, SHG imaging was employed to assess stromal collagen remodelling following dual treatment with Gemcitabine and STAT3 inhibitors in a mouse model of pancreatic cancer [38], while Gemcitabine delivery upon stromal intervention/reduction was monitored using dual SHG and fluorescence doxorubicin imaging [12, 39]. In line with these studies, recent SHG imaging of a human pancreatic tissue microarray (>80 patients) revealed a positive correlation between collagen abundance, tumour stage and resistance to chemotherapy [14]. Imaging of intrinsic tissue auto-fluorescence can also enable visualisation of other ECM components such as elastin in this context [40].

The metabolic activity of cancer can be observed using fluorescence lifetime imaging (FLIM) of cellular NADH and FADH fluorescence (ratio of free to bound NADH) [41]. A number of studies have used this approach for label-free *in-vivo* identification of cancerous or precancerous lesions, which are applicable to recent studies of pancreatic cancer metabolism [42]. Moreover, visualisation of other stromal components such as tumour vasculature has been achieved using quantum dots (QD). Here, QD imaging in a live xenograft model of pancreatic cancer provided invaluable information regarding drug targeting of cancer cells in relation to the proximity to blood vessels [43] and supported the hypothesis that enhancing tumour vasculature patency may improve drug penetrance in pancreatic cancer tissue [12, 13]. Engineering stromal and cancer cells to express fluorescent reporters has also been employed to directly visualise the cross-talk between cancer cells and their surrounding stroma. For instance, colour coding of cancer and stromal cells implicated stellate cells on the onset of angiogenesis and on colonisation of distant organs [44]. Furthermore, in an elegant study, Yang et al [45] implanted RFP-pancreatic cancer cells in a GFP-expressing host to directly visualise tumour-stroma interactions and drug response of both cancer and stromal cells. Imaging of the tumour microenvironment therefore allows us to embrace the contextual complexity of pancreatic cancer and fine-tune how to best modulate the pancreatic tumour-associated stroma within the confines of normal tissue architecture and organ function.

Live Imaging of Biosensors to Monitor the Dynamics of Tumour Cell Signalling

The development of fluorescent biosensors has enabled us to dissect the dynamics of molecular events and provided insights into their spatio-temporal regulation. As such, imaging of biosensors has shed light on mechanisms occurring in pancreatic cancer *in vivo* such as changes in cell proliferation, survival, invasion, metastasis and response to chemotherapy. For example, live imaging of the prototypical RhoGTPases, RhoA and Rac-1, which are known to drive cancer cell migration, has been achieved using Förster Resonance Energy Transfer (FRET) biosensors and revealed a subcellular regulation of the small GTPases at the leading edge of invading cells *in vitro* and *in vivo,* therefore increasing our understanding of actomyosin cytoskeletal remodelling and cell motility in pancreatic cancer [46, 47]. Similarly, an extra-cellular FRET MMP-11 biosensor substrate was developed to track and characterise MMP-11 mediated invasive mechanisms in pancreatic cancer cells [48], while live monitoring of cell-cell adhesion dynamics upon anti-migratory drug treatment was recently assessed using a Fluorescence Recovery after Photobleaching (FRAP) biosensor to monitor E-cadherin stability in pancreatic cancer [49].

Live imaging of fluorescent biosensors is an emerging pre-clinical tool for cancer research and has helped probe other aberrant events in cancer such as alterations in cell proliferation, survival, metabolic events and cell signalling. For instance, various probes such as Fucci sensors, CDK1-FRET biosensors and photo-marking H2B-Dendra reporters are used to elucidate the dynamics of cell proliferation and give insights on the efficacy of anti-proliferative drugs [50-52]. Similarly, the use of Akt, Erk and PAK -FRET biosensors have permitted us to untangle the molecular mechanisms governing cell survival and signal transduction *in vivo* [53, 54]. More recent developments may provide further insights into signalling pathways in pancreatic cancer. For example, polarisation resolved imaging of homo-FRET between identical fluorophores can visualise clustering of molecules such as glycosylphosphatidylinositol (GPI)-anchored proteins or epidermal growth factor receptor (EGFR), both of which commonly feature as deregulated signalling nodes in cancer [55, 56]. Multiplexed imaging of several FRET biosensors in a single cell using spectral unmixing or homo-FRET based biosensors [57] can help us probe the spatio-temporal dynamics of intertwined signalling events which often involve complex molecular feedback loops. This approach can allow us to best evaluate pre-clinical treatments regimes by circumventing regulation loop that may lead to chemoresistance. For a detailed and non-exhaustive list of biosensors and fluorescent techniques used to image cancer, see [58] figure 2 which provides an example of FRET imaging to monitor Src activity in live tumours. We suggest that using these tools for future pancreatic cancer research will rapidly expand our understanding of the molecular events occurring during pancreatic cancer progression and therapeutic intervention.

Multi-Modal Imaging: a Pre-Clinical Tool in Pancreatic Cancer

Simultaneous imaging of different aspects of pancreatic cancer provides a detailed picture of cancer response to pre-clinical strategies and is likely to facilitate therapeutic discovery in the disease [35, 51]. In an elegant study, a fluorophore-labelled lectin antibody was administered in mice bearing pancreatic tumours and used in combination with immunohistochemical analyses to image the effect of combination therapy on tumour vasculature [13]. Likewise, Wang et al designed a Gemcitabine-loaded magnetic albumin nanosphere to conduct simultaneous targeted chemotherapy and MRI imaging of drug delivery [59]. These approaches allow us to assess the level of drug penetration into cancer tissue. Integrating multi-modal imaging technologies has also been employed to monitor drug targeting in a dynamic, context-dependent, single and subcellular level. As such, intravital imaging has been used to monitor the intracellular pharmacokinetics of PARP-1 and microtubule inhibitors and underlines the heterogeneity of tumour response to chemotherapy [60, 62]. Interestingly, longitudinal imaging using surgically implanted imaging

windows allows us to integrate the spatio-temporal and contextual complexity of cancer progression. In particular, this technique was used to characterise the formation of a metastatic niche during liver colonisation by cancer cells, as well as monitor live events within the abdominal body cavity including *in situ* pancreatic biology in real-time [63]. Importantly, this new approach will enable us to monitor drug kinetics in real-time within the same mouse (pre- and post- dosing) and is set to provide a reliable new tool for future therapeutic intervention studies in pancreatic cancer (**Figure 1**).

Application of Novel Imaging Technologies in the Clinic for Pancreatic Cancer

One such example is developed by VisEn Medical Inc, where the injection of a proteolytically activated fluorophore coupled with fiberoptic confocal microscopy allows highly sensitive characterisation of tumour stage, lymph node status and has also been used for fluorescence-guided surgery [64]. Lastly, a surface-enhanced resonance Raman scattering (SERRS) nanoparticle has been used to detect macroscopic pancreatic lesions to identify tumour margins, and therefore represents a promising tool for precise pancreatic cancer imaging and resection [65].

Conclusions

It is clear that the tumour microenvironment is highly complex and future therapies will likely require a multicellular and multi gene targeting approaches. Nanotechnology advancements hold potential to enhance both the delivery and specific targeting of pancreatic cancer, while state-of-the art imaging technologies increase our understanding of the biology of the disease and facilitate the discovery of new treatments. We have described promising preclinical imaging technologies, which will improve our understanding of the disease and help guide the development of therapeutic strategy. In conclusion, marriage of nanoparticle delivery with advanced molecular imaging is set to rapidly improve the management and future treatment of pancreatic cancer.

Figure legends

Figure 1. Strategies to Visualise and Overcome Barriers to Therapeutics in Pancreatic Cancer. There are currently several barriers to effective drug delivery in pancreatic tumours. 1. Pancreatic stellate cells, are responsible for orchestrating fibrosis and feed pro-survival signals to tumour cells. Simultaneous targeting of both tumour and stellate cells is essential in pancreatic cancer treatment. 2. Chemoresistance and genetic

heterogeneity of pancreatic cancer cells makes it extremely difficult to treat all patients with a single approach. Multi-target approaches using RNA interference (RNAi) therapeutics can help overcome this problem. RNAi therapeutics can inhibit any target at the gene level and could be delivered using nanoparticles. These can be delivered using nanoparticles, which can also be tailored to specifically target tumour cells/stellate cells. RNAi therapeutics can be combined with nanoparticle vehicles and advanced genomics to deliver personalised medicine based on the genetics of a patient's tumour, with high efficacy and minimal off-target toxicity. 3. Fibrosis can act as a physical barrier to drug penetration. It distorts tumour vasculature resulting in a hypoxic microenvironment, driving chemoresistance and metastases. To overcome this barrier, nanoparticles can be used to bypass fibrosis and reach tumour cells. Stromal remodelling strategies can also enhance drug access to tumour cells. Preclinical imaging approaches of the pancreatic tumour microenvironment offer insight into the molecular basis of pancreatic cancer and can improve the development of new therapies in this disease. (A) Live SHG imaging of collagen I fibers surrounding metastatic pancreatic cancer cells in an intrasplenic model of liver metastasis. (B) Intravital imaging of blood vasculature in a subcutaneous xenograft model of pancreatic cancer using quantum dot imaging [43].

Figure 2: Live Intravital Imaging of Pancreatic Cancer Signaling: Monitoring Src Kinase Activity using FRET Imaging. FRET imaging of a Src biosensor in a xenograft model of pancreatic cancer with or without dasatinib treatment. Representative lifetime maps of "ON" cells (top panel, control treatment) and "OFF" cells (bottom panel, dasatinib treatment) [43].

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Table 1. Nanoparticle-based therapies in clinical use for cancer.

Composition	Trade Name	Disease	Administration	Reference
Liposomal doxorubicin	Myocet	Combination therapy with cyclophosphamide in metastatic breast cancer	Intravenous	[67]
Liposomal-PEG doxorubicin [#]	Doxil/Caelyx	HIV-related Kaposi's sarcoma, metastatic breast cancer, metastatic ovarian cancer	Intramuscular	[68-70]
Albumin-bound paclitaxel	Abraxane*	Metastatic breast cancer, **metastatic pancreatic cancer**	Intravenous	[71, 72]
Methoxy-PEG-poly(D,L-lactide) taxol	Genexol-PM	Metastatic breast cancer	Intravenous	[73]
PEG-L-asparaginase	Oncaspar	Acute lymphoblastic leukaemia	Intravenous, intramuscular	[74]

* The field of nanotherapies has exploded in the last five years, resulting in the use of nanoparticle-based therapies in the treatment of several malignancies. Albumin-bound paclitaxel is currently employed as a therapy for pancreatic cancer. There is ongoing work by several groups towards establishing the ideal nanoparticle for use in the treatment of pancreatic cancer. [#]PEG, Polyethylene glycol.

Table 2. Current clinical trials testing nanoparticle therapies in cancer.

Composition	Trade Name	Disease	Administration	Status	Reference	Clinical Trial Number
Liposomal doxorubicin	Doxil	Soft tissue sarcoma	Intravenous	Phase I/II	[75]	NCT00949325
Polyglutamate	Xyotax	Metastatic	Intravenous	Phase II	[76]	NCT00265733

paclitaxel		breast cancer				
PEG-camptothecin[#]	MAG-CPT (PNU 166148)	Advanced solid cancers	Intravenous	Phase I	[77]	NCT00004076

[#]PEG, Polyethylene glycol.

FIGURE 1

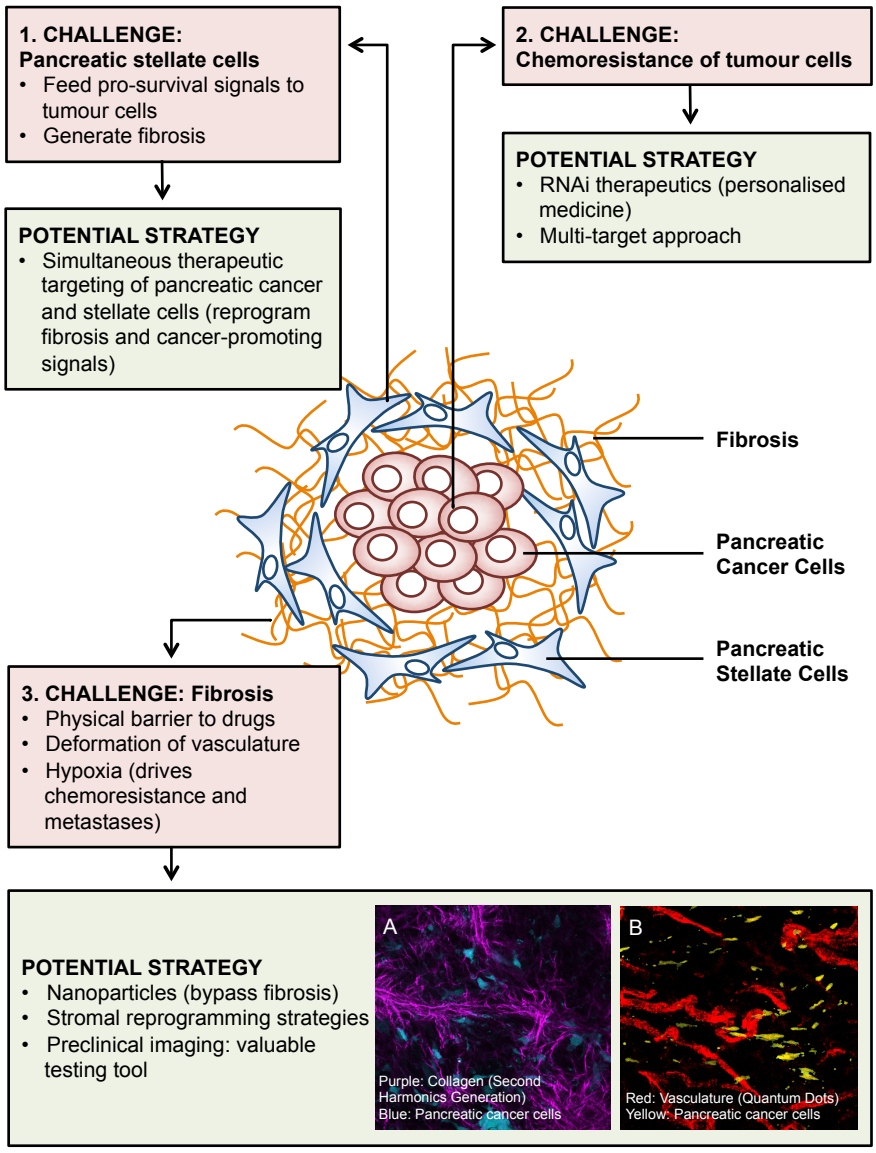
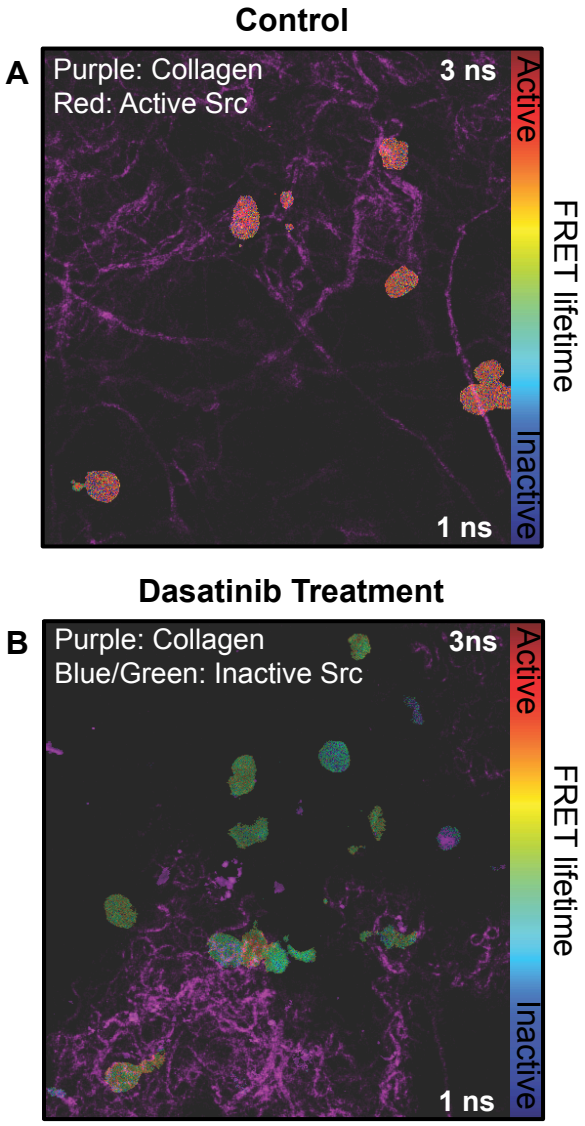

1. CHALLENGE:
Pancreatic stellate cells
• Feed pro-survival signals to tumour cells
• Generate fibrosis
POTENTIAL STRATEGY
• Simultaneous therapeutic targeting of pancreatic cancer and stellate cells (reprogram fibrosis and cancer-promoting signals)
2. CHALLENGE:
Chemoresistance of tumour cells
POTENTIAL STRATEGY
• RNAi therapeutics (personalised medicine)
• Multi-target approach
Fibrosis
Pancreatic Cancer Cells
Pancreatic Stellate Cells
3. CHALLENGE: Fibrosis
• Physical barrier to drugs
• Deformation of vasculature
• Hypoxia (drives chemoresistance and metastases)
POTENTIAL STRATEGY
• Nanoparticles (bypass fibrosis)
• Stromal reprogramming strategies
• Preclinical imaging: valuable testing tool
A
Purple: Collagen (Second Harmonics Generation)
Blue: Pancreatic cancer cells
B
Red: Vasculature (Quantum Dots)
Yellow: Pancreatic cancer cells

FIGURE 2


Control
A
Purple: Collagen
Red: Active Src
3 ns
1 ns
Active
Inactive
FRET lifetime
Dasatinib Treatment
B
Purple: Collagen
Blue/Green: Inactive Src
3ns
1 ns
Active
Inactive
FRET lifetime