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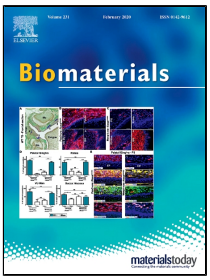

Targeting the Undruggable in Pancreatic Cancer Using Nano-Based Gene Silencing Drugs

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PII:	S0142-9612(19)30860-9
DOI:	https://doi.org/10.1016/j.biomaterials.2019.119742
Reference:	JBMT 119742
To appear in:	*Biomaterials*
Received Date:	01 August 2019
Accepted Date:	25 December 2019

Beilin, Kynan Feeney, Nan Q. Nguyen, Andrew R. Ruszkiewicz, Chris Worthley, John Chen, Mark E. Brooke-Smith, Virginia Papangelis, Andrew D. Clouston, Patrick Martin, Andrew P. Barbour, Thomas J. O'Rourke, Jonathan W. Fawcett, Kellee Slater, Michael Hatzifotis, Peter Hodgkinson, Mehrdad Nikfarjam, James R. Eshleman, Ralph H. Hruban, Christopher L. Wolfgang, Mary Hodgin, Aldo Scarpa, Rita T. Lawlor, Stefania Beghelli, Vincenzo Corbo, Maria Scardoni, Claudio Bassi, Andrew V. Biankin, Judith Dixon, Craig Nourse, Nigel B. Jamieson, Targeting the Undruggable in Pancreatic Cancer Using Nano-Based Gene Silencing Drugs, *Biomaterials* (2019), https://doi.org/10.1016/j.biomaterials.2019.119742

Targeting the Undruggable in Pancreatic Cancer Using Nano-Based Gene Silencing Drugs

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Abstract

Pancreatic cancer is predicted to be the second leading cause of cancer-related death by 2025. The best chemotherapy only extends survival by an average of 18 weeks. The extensive fibrotic stroma surrounding the tumor curbs therapeutic options as chemotherapy drugs cannot freely penetrate the tumor. RNA interference (RNAi) has emerged as a promising approach to revolutionize cancer treatment. Small interfering RNA (siRNA) can be designed to inhibit the expression of any gene which is important given the high degree of genetic heterogeneity present in pancreatic tumors. Despite the potential of siRNA therapies, there are hurdles limiting their clinical application such as poor transport across biological barriers, limited cellular uptake, degradation, and rapid clearance. Nanotechnology can address these challenges. In fact, the past few decades have seen the conceptualization, design, pre-clinical testing and recent clinical approval of a RNAi nanodrug to treat disease. In this review, we comment on the current state of play of clinical trials evaluating siRNA nanodrugs and review pre-clinical studies investigating the efficacy of siRNA therapeutics in pancreatic cancer. We assess the physiological barriers unique to pancreatic cancer that need to be considered when designing and testing new nanomedicines for this disease.

Key words

Pancreatic cancer, Nanomedicine, Nanoparticles, RNA-interference, small interfering RNA

Introduction

Pancreatic ductal adenocarcinoma [referred to as pancreatic cancer (PC)] is the fourth leading cause of cancer-related deaths in developed countries with a dismal five-year survival rate of 8% [1]. PC has seen little improvement in patient survival in the past four decades and is projected to be the second leading cause of cancer mortality by 2025 [2]. Unfortunately, PC is often diagnosed at an advanced stage with the development of metastatic spread at diagnosis [3]. Surgical resection improves patient survival, but only 15-20% of patients have surgically resectable tumors and long-term survival after surgery remains poor [3, 4]. Tragically, our best chemotherapy treatments only improve life by an average of 8-16 weeks [4] and there is an urgent need to develop more effective treatments.

One of the defining histopathological features of PC is the highly fibrotic stroma that can constitute more than 80% of the tumor mass [5, 6] (**Figure 1**). Importantly, a higher stromal content in human PC patients is associated with poor survival outcome [7, 8]. This desmoplastic reaction results in the deposition of an unusually dense network of extracellular matrix proteins around tumor elements, which compresses and distorts tumor blood vessels and acts as a physical barrier to chemotherapy drug delivery [9-11]. In addition, this abnormal vasculature drives hypoxia in PC tumors which promotes the development of chemoresistance [9]. This dense fibrosis is produced by cancer associated pancreatic stellate cells (PSCs) which are normally in a quiescent form in healthy pancreas but are recruited by PC cells where a cross-talk mechanism fuels the aggressiveness of PC [9, 12-16]. Indeed, PSCs are now considered key cellular therapeutic targets in order to reprogram the fibrosis in PC and also to block the bi-directional pro-tumorigenic signaling that exists with cancer cells. It is thus imperative to consider both the tumor and its surrounding stroma when designing novel therapeutic strategies for PC. In this regard, there has been intense research to try and harness the power of the RNA interference (RNAi) gene silencing mechanism in both tumor cells and stromal cells to therapeutically inhibit tumor-promoting genes. RNAi molecules including small interfering RNA (siRNA) can be designed to silence the expression of genes whose proteins are considered difficult to inhibit using chemical agents or monoclonal antibodies. This technology offers the opportunity to target a cocktail of tumor-promoting genes in different cell types present in the tumor microenvironment. However, despite the potential of siRNA-based therapies, the challenge of delivery and release of siRNA into cells are obstacles which hinder its full clinical potential. To overcome these hurdles, nanotechnology represents a promising way to deliver siRNA to cells. In fact, an increasing number of studies have investigated the use of non-viral nanoparticles to deliver siRNA to PC tumors in pre-clinical mouse models [17]. In this review, we discuss the prospects and challenges of utilizing nanoparticles as a delivery vehicle for siRNA in PC (**Figure 1**). Furthermore, we comment on the physiological barriers unique to PC that need to be addressed when designing new nanotherapeutic drugs for this devastating disease.

Targeting the "Undruggable" Using Gene Silencing Drugs

In the past decade, research has identified a wealth of novel cancer-related genes that promote tumor progression, metastases and treatment resistance in both PC tumor and surrounding stromal cells [18]. Many of these genes and proteins are considered 'undruggable' since they do not have pharmacological inhibitors or are difficult to inhibit using small drug molecules due to: 1) a lack of well-defined ligand binding sites; or 2) close amino acid sequence homology with other proteins which limits target selectivity. The potential to selectively inhibit these genes using RNAi-nanomedicines represents a highly promising strategy to halt tumor progression and improve overall patient survival.

RNAi is a naturally occurring gene silencing mechanism in mammalian cells which can be used to inhibit therapeutic gene targets [19, 20]. In contrast to pharmacological inhibitors that are often not specific to their target gene, RNAi molecules such as siRNA or short hairpin RNA (shRNA) offer the advantage of greater selectivity due to their mechanism of action [21]. siRNA consists of double-stranded RNA of approximately 21-23 base pairs with 2-3 nucleotide overhangs at the 3′ end. It binds to the RNA-induced silencing complex (RISC) located in the cell cytoplasm, where the guide strand of siRNA directs the RISC protein complex to recognize and cleave target mRNA between nucleotides 10 and 11 upstream of the 5′ end of siRNA, resulting in its cleavage and degradation [21-23] (**Figure 2**). Once cleavage has taken place the RISC-siRNA can be recycled for further cleavage reactions. Thus, the ability of siRNA to silence the expression of any gene has led to a major effort to harness its power for the treatment of many types of human disease such as cancer.

Despite the promise of siRNA-therapeutics for cancer treatment, delivery of siRNA into cells is a major obstacle preventing its use in the clinic. This is due to: (1) large size of siRNA (approx. 13.5 kDa) and its negative charge; (2) naked (unmodified) siRNA is prone to degradation by serum proteins in the blood, and can be rapidly taken up and eliminated from the body by the reticuloendothelial system [24]. As mentioned above, the dense fibrotic stroma and vascular barriers present in PC tumors add a layer of extra complexity for effective siRNA delivery to PC cells. To overcome these hurdles, non-viral nanoparticles are being used to package and deliver siRNA to cells [17].

Nanoparticles as a Delivery Vehicle for siRNA

Non-viral nanoparticles can act as delivery vehicles for a host of different therapeutic drugs [25]. Indeed, nano-based medicines are already in clinical use for the treatment of cancer. Nanoparticles can be designed with physical properties which make them attractive delivery vehicles for drugs including: 1) sub-micrometer size; 2) high surface-to-volume ratio; 3) potential to chemically modify their surface with tumor cell targeting moieties or attach polyethylene glycol (PEG) which helps provide stability as well as increase blood circulation time; and 4) versatility to package and deliver proteins, small molecule inhibitors, chemotherapy drugs or nucleic acids [26]. The last 20 years has seen the design and synthesis of many different types of non-viral nanoparticles made from a variety of compounds including polymers, lipids, aptamers and inorganic materials to deliver siRNA to cells [27].

To provide nanoparticles the best opportunity to penetrate and accumulate within solid tumors, they are typically synthesized in a size range of 10-200 nm. This size enables nanoparticles to take full advantage of the ‘enhanced permeability and retention effect’ (EPR) which occurs due to the poorly formed and often leaky disorganized vessels within a solid tumor [28]. Nanoparticles larger than 10 nm have difficulty in penetrating healthy tissue due to well-developed and functional vessels which possess tight gap junctions [29]. In a solid tumor the presence of leaky vessels with dysregulated large gap junctions combined with poor lymphatic drainage allow nanoparticles to accumulate and become trapped within the tumor [30]. This phenomenon is referred to as ‘passive tumor targeting’. Although efficiency of nanoparticle delivery via the EPR effect is debated, a recent study in humans [31] showed for the first time that a chemotherapy drug (Camptothecin) conjugated to a biocompatible co-polymer nanoparticle comprising of cyclodextrin and polyethylene glycol (PEG) with a size of 20-30 nm (CRLX101, Cerulean Pharma Inc) was able to penetrate into human gastric tumors which were collected via endoscopy. Notably, no drug-nanoparticle was detected in adjacent non-tumor tissue implying that the nanoparticle was able to passively accumulate into the tumor due to the EPR effect. This is encouraging, and as scientists we need to be cautious that we

utilize the best mouse tumor models which mimic the heterogeneity of the altered vasculature and microenvironment that contribute to the EPR effect. Importantly, all current nanomedicines used in the clinic passively target tumors. Examples include, Doxil® (liposomal doxorubicin) which was the first FDA approved nanomedicine to enter the clinic and Abraxane™ (albumin bound paclitaxel) which is used in first line therapy for PC [32]. Another nanomedicine clinically approved for second line treatment of metastatic PC is Onivyde which comprises of the topoisomerase I inhibitor irinotecan encapsulated in a liposomal nanoparticle decorated with polyethylene glycol (PEG) which helps increase stability and circulation time in the bloodstream [33]. While these two agents have been successful in delivering chemotherapeutics to pancreatic tumors, there are several additional challenges that need to be considered when designing nanoparticle systems for therapeutic siRNA delivery.

An important physical requirement that requires careful consideration when designing nanoparticles for siRNA delivery is its ability to release siRNA into the cytosol. Once a nanoparticle-siRNA complex reaches a tumor, it must be internalized by the cells and escape from early endosomes to allow siRNA to engage RISC [34] (**Figure 2**). Recently, it has come to attention that escape of siRNA from endosomes is not a trivial process but a key determinant for effective gene silencing activity [35, 36]. Nanoparticles carrying siRNA interact with the cell membrane of cancer cells to trigger endocytosis. The size, shape, charge and surface chemistry of a nanoparticle greatly influence the mechanism(s) of endocytosis [reviewed in detail elsewhere [37]]. Once internalized, nanoparticle-siRNA trapped in early endosomes undergo intracellular trafficking which is a dynamic process [38]. Early endosomes transport their cargo to different subcellular destinations. Some of the cargo will also be recycled to the plasma membrane via recycling endosomes and exocytosed from the cell, while other early endosomes will mature into late endosomes which integrate with lysosomes to form endolysosomal vesicles [38]. Hydrolytic enzymes within these vesicles will degrade the remaining cargo [38]. It has been suggested that the buffering capacity of nanoparticles with a positive surface charge activate proton pumps which increase osmotic pressure inside early endosomes resulting in swelling and rupture (termed, proton-sponge effect) [39]. Another possible mechanism for siRNA endosomal escape is cationic lipids present in nanoparticles fuse with anionic lipids in the plasma membrane of endosomes causing membrane disruption [40]. However, despite these routes for siRNA escape, a study by Gilleron et al [41] demonstrated that lipid nanoparticles (LNPs) which belong to a class of highly advanced nanoparticle delivery systems for siRNA have low efficiency (approx. 1-2%) for escape from early endosomes. Another study showed that up to 70% of LNPs internalized into cells are exocytosed by late endosomes / endolysosomes after 24 hours [42]. These studies highlight the importance of understanding how different nanoparticle systems are not only internalized into cells but how effectively they release their cargo to achieve potent gene silencing activity. This is an area of active research with the design and synthesis of next generation polymeric, lipid, inorganic or hybrid nanoparticles [43-45]. Below are some recent examples demonstrating the potential of these nanoparticles for effective siRNA uptake and release into cells.

Lipid and polymeric-based nanoparticles: Lipid-based nanoparticles (LNPs) which contain pH-responsive ionizable cationic lipids have been demonstrated to be highly efficient for the delivery and release of siRNA into cells. These lipids are amphiphilic and can efficiently self-assemble with siRNA via an electrostatic interaction under acidic conditions [46]. Importantly, these lipids have a near neutral surface charge when complexed to siRNA at physiological pH 7 and display a low toxicity and immunogenic profile. However, when the lipids are exposed to a low pH acidic environment present in early / late endosomes they become positively charged which encourages nanoparticle-siRNA disassembly and endosomal membrane

disruption to allow siRNA to escape into the cytoplasm. Incorporation of these lipids into nanoparticles has seen a marked improvement in siRNA gene silencing activity. One of the earliest studies reported by Lee et al [47] showed that LNPs which contained the ionizable lipid 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-KC2-DMA) were able to potently silence the expression of the androgen receptor in orthotopic prostate tumors in mice. This led to a marked decrease in prostate specific antigen levels in the blood. Jyotsana et al [48] demonstrated LNPs which incorporated the ionizable lipid DLin-MC3-DMA were able to deliver siRNA targeted against the fusion oncogene BCR-ABL with high efficiency to human Chronic Myeloid Leukemia (CML) cells both *in vitro* and *in vivo*. These cells are notoriously difficult to transfect using standard cationic nanoparticles thus highlighting the advantages of incorporating ionizable lipids into nanoparticles.

Polymeric nanoparticles comprised of ionizable polymers have also shown great promise as siRNA delivery vehicles. For example, 7C1 ionizable polymeric nanoparticles preferentially target the endothelium and can safely deliver very low amounts of siRNA with potent gene silencing activity *in vivo*. Dahlman et al [49] reported that 7C1 nanoparticles containing 0.1 mg/kg siRNA could silence a target gene expressed in the lung endothelium by 90% in mice. Notably, 7C1 nanoparticles could deliver up to 5 different siRNAs to achieve potent multi-gene silencing *in vivo*. Recently, the clinical potential of 7C1 nanoparticles was further highlighted by potent gene silencing in the endothelium of multiple organs in non-human primates without inducing any toxicity or immune response [50].

Inorganic-based nanoparticles: Carbon nanotubes and gold nanoparticles have been used as highly effective delivery vehicles for siRNA. Cao et al [51] recently developed a novel single-wall carbon nanotube (SWCNT) which could package and deliver both a chemotherapy drug and siRNA simultaneously to tumor cells. The surface of the SWCNT was modified with polyethyleneimine (PEI) covalently linked with betaine to produce a pH responsive SWCNT. Moreover, the cell penetrating peptide BR2 was conjugated to the surface to encourage cell uptake. The multifunctional nanoparticle was able to be internalized into tumor cells and effectively release siRNA from the endosomes together with a chemotherapy drug to significantly inhibit tumor growth in mice. Perche et al [52] synthesized gold nanoparticles functionalized with PEG to deliver siRNA to HeLa cells. Like most gold nanoparticles coated with PEG, endosomal escape of siRNA was limited. However, Perche et al [52] conjugated hydroxychloroquine, a clinically approved drug with endosomal disrupting properties, to the surface of the gold nanoparticles. This improved the release of siRNA from early endosomes which correlated to a two-fold increase in gene silencing activity.

Hybrid-based nanoparticles: Hybrid nanoparticles are also showing promise for the delivery of siRNA to cells. Qiu et al [53] recently developed a highly novel hybrid nanoparticle system which comprised of two cationic polymers, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 1,2-dioleoyl-sn-glycero-3-phoshoethanolamine (DOPE) as well as cholesterol to self-assemble siRNA into a cationic nanocomplex. These nanoparticles are prone to rapid exocytosis and degradation once internalized into cells via the endosome-lysosomal degradation pathway [54]. To alter the intracellular trafficking of the nanoparticles, the authors decorated their surface with an endoplasmic reticulum (ER) membrane isolated from cancer cells. This led to a change in the mode of cellular uptake for the nanoparticles as well as intracellular trafficking via the endosome-Golgi-ER pathway. Importantly, this allowed the nanoparticle-siRNA complex to escape lysosomal degradation and resulted in a pronounced increase in gene silencing activity *in vitro* and *in vivo*.

Current State of Play of Gene Silencing Nanodrugs in the Clinic

Despite the numerous challenges, the past decade has seen an increased number of RNAi-based nanotherapeutics progress from pre-clinical studies to clinical trial. In particular, the development of LNPs has led to the first FDA approved RNAi therapeutic (Patisiran, ONPATTRO®) [55]. In brief, LNPs are non-viral nanoparticles with a size of approximately 80-100 nm and consist of a mixture of cationic, ionizable and PEGylated lipids [56]. These nanoparticles have properties that make them highly desirable for use in the clinic, namely, low surface charge which minimizes toxicity and immunogenicity, high drug encapsulation efficiency and reproducible methodology that allows for large scale clinical grade synthesis. LNPs were used to encapsulate siRNA targeting wild type and mutant transthyretin (TTR), a disease-causing gene for hereditary transthyretin (TTR)-mediated amyloidosis autosomal dominant disorder (**Table 1**). Treatment options for this genetic disease were limited. Patisiran® delivered intravenously at 0.3 mg/kg once every three weeks was able to potently silence the expression of TTR in liver hepatocytes (target cell) leading to a significant reduction of TTR protein in the blood and tissue [57, 58]. This correlated with a marked improvement in disease symptoms. The approval of Patisiran® as a RNAi therapeutic for human disease provides a much-needed boost of confidence for RNAi researchers and nanotechnologists and demonstrates proof-of-principle that the RNAi gene silencing mechanism can be harnessed to therapeutically inhibit an undruggable gene to achieve a positive clinical outcome.

CALAA-01, produced by Calando Pharmaceuticals, is a targeted polymer nanoparticle-siRNA system which was evaluated in clinical trial for the treatment of cancer. This was the first RNAi therapeutic to be administered to cancer patients. CALAA-01 contained a linear cyclodextrin based polymer which could self-assemble siRNA via a simple mixing process as well as a hydrophilic polymer [(adamantane polyethylene glycol (AD-PEG)] to provide nanoparticle stability in blood. A cancer cell targeting peptide was conjugated to its surface, designed to bind the human transferrin receptor and undergo receptor-mediated endocytosis. Importantly, the investigators were able to show that the nanoparticle-siRNA, when administered to human cancer patients, was able to penetrate solid tumors and silence the expression of its target gene (ribonucleotide reductase M2, RRM2) [19, 59, 60]. However, the phase 1 clinical trial was terminated when several patients experienced dose limiting toxicities including diarrhea, fever, and fatigue [60]. The cause was most likely due to nanoparticle instability and breakdown of the individual components in the blood. Toxicity was thought to have arisen from the transferrin targeting peptide. This study highlights the complexity of nanoparticle systems when comprised of multiple components and the potential to breakdown when exposed to circulation in blood. Despite the termination of this potential RNAi therapeutic, important lessons can be taken from this highly valuable study to produce 'next generation' nanoparticle systems for the delivery of siRNA to solid tumors. To date, more than 20 RNAi-based nanomedicines have undergone or are currently in clinical trials for treatment of multiple cancer types (**Table 2**).

Gene Silencing Nanomedicines for the Treatment of Pancreatic Cancer

There are an increasing number of studies reporting the development and use of RNAi therapeutics for PC. In 2007, Pirollo et al [61] demonstrated the potential of liposomes containing human epidermal growth factor receptor 2 (HER-2) siRNA with a transferrin receptor antibody attached to its surface. Following intravenous injection into mice with subcutaneous PC tumors, HER-2 protein expression was reduced along with a marked decrease in tumor growth [61]. Other studies have also investigated a range of different nanoparticle systems packaged with siRNA against tumor promoting genes including, CUX-1 (cut like

homebox 1), VEGF (vascular endothelial growth factor) and EPAS1 (endothelial PAS domain protein 1) *in vivo* (summarised in **Table 3**) [62-66]. Notably, several studies have highlighted the ability of RNAi nanomedicines to selectively target and inhibit the undruggable mutant KRAS gene in PC tumors. This gene is present in over 90% of PC tumors [67]. The high degree of selectivity for RNAi was showcased when shRNA was able to inhibit the mutant KRAS gene without effecting the expression of wild-type KRAS in mouse PC tumors [68]. This led to a significant reduction in tumor growth without any toxicity to non-tumor cells.

In addition, some studies have developed multi-functional nanoparticles capable of dual siRNA and chemotherapy drug delivery. A recent study used liposomes to deliver both gemcitabine and siRNA targeting RRM2 to subcutaneous PC tumors [69]. Given that RRM2 can promote gemcitabine resistance, knockdown of RRM2 in tumors with high expression of RRM2 led to a further reduction in tumor growth with liposomal gemcitabine delivery [69]. Similarly, Yin et al [70] developed a gold nanorod system to deliver both KRAS siRNA and doxorubicin to subcutaneous PANC1 tumors. Importantly, these nanoparticles were activated by near-infrared light (655 nm) both *in vitro* and *in vivo* which stimulated the nanoparticles to release the siRNA and doxorubicin [70]. Another study published by the same group used light-activated 2D graphene oxide nanosheets for dual delivery of siRNA targeting both histone deacetylase 1 (HDAC1) and KRAS in subcutaneous pancreatic tumors [71]. While these light-activated nanoparticles appear promising, it remains to be investigated whether these nanoparticles will be effective in an orthotopic tumor setting and whether they can be applied to metastatic disease. Another example of a multifunctional nanoparticle was developed by Yoo et al [72], utilising a magnetic nanoparticle to both monitor treatment with MRI imaging and delivery of siRNA targeting PD-L1 in subcutaneous pancreatic tumors. An elegant study published by Boehnke et al [73] detailed the design and *in vivo* testing of a novel theranostic nanoparticle for both diagnostic and siRNA delivery capabilities. This nanoparticle consisted of a liposomal core with layer-by-layer assembly of a biosensing peptide, a targeting peptide and siRNA encapsulation. Impressively, the biosensing peptide is cleaved by proteases expressed in the tumor microenvironment and releases a synthetic reporter that can be detected in urine, as demonstrated in three different mouse models of pancreatic, ovarian and colorectal cancer [73]. Collectively, these studies provided important proof-of-concept for the use of siRNA therapeutics in the treatment of PC, however many of the preclinical models utilized subcutaneous PC tumors in mice. Unfortunately, these tumors lack many of the drug delivery challenges associated with pancreatic tumors in the clinical setting i.e. presence of an extensive fibrotic stroma, dysfunctional or compressed blood vessels and a functional immune system. Therefore, in recent times, a stronger emphasis has been placed on examining the therapeutic potential of RNAi nanomedicines using more clinically relevant PC models.

One of the earliest studies using an orthotopic PC mouse model to examine a siRNA-nanotherapeutic was performed by Zhao et al [74]. This model involves the surgical implantation of human or mouse PC cells into the tail of the pancreas. The nanoparticles were comprised of a cationic hydrophobic co-polymer core that encapsulated the chemotherapy drug gemcitabine (used in the first line treatment of PC) and electrostatically bound siRNA targeting hypoxia inducible factor-1α (HIF-1α) on its surface [74]. The nanoparticles were also coated in a PEGylated lipid bilayer to help minimize the interaction with serum proteins and maximize circulation time. Systemic administration of the RNAi-drug induced potent HIF-1α knockdown at the protein level as well as a marked reduction in tumor volume [74]. Our research program showed that polymeric star-shaped nanoparticles consisting of poly(dimethylaminoethyl methacrylate) (PDMAEMA) and poly(ethylene glycol) methacrylate (POEGMA, a homologue of PEG) were able to rapidly self-assemble siRNA and deliver it with high efficiency to

orthotopic PC mouse tumors to silence the expression of an undruggable gene (βIII-tubulin) which plays an important role in regulating PC growth and chemosensitivity [75]. A study by Mahajan et al [76] demonstrated profound anti-tumor effects of a novel theranostic nanoparticle delivering siRNA targeted against Polo-like kinase 1 (PLK1) a mitotic kinase known to be highly expressed in PC cells and is a major regulator of the cell cycle [77]. The significance of this study was highlighted by using both the PC syngeneic and genetically engineered mouse model (GEMM). Both models have a PC tumor microenvironment that closely mimics the human setting [76]. Importantly, both models also have a fully functional immune system. A recent study demonstrated an anti-tumor and metastatic effect of inorganic gold nanoparticles delivering nerve growth factor (NGF) siRNA to a patient-derived xenograft PC tumor in mice [78]. Taken together, these studies clearly provide strong rationale for the continued development of gene-silencing nanomedicines for the treatment of PC.

RNAi nanotherapeutics also has potential use for targeting the non-tumor cell population within the PC tumor microenvironment. Pancreatic Stellate Cells (PSCs) are the key cell type responsible for producing the dense fibrotic stroma in PC tumors [5, 6]. Gold nanoparticles with PEGylated lipids were recently designed to package all-trans retinoic acid (ATRA) and siRNA targeting heat shock protein-47 (HSP47) [79]. ATRA, an active metabolite of vitamin A, and siRNA against HSP47 are both known to induce PSC quiescence [80-82]. Notably, systemic administration of the nanoparticle into mice with orthotopic PC tumors (established via injection of cancer cells and PSCs into the pancreas) resulted in a significant remodelling of the fibrotic tissue. This led to increased chemotherapy drug penetration and inhibited PC tumor growth and metastases. This study demonstrates the potential of combining RNAi therapeutics with chemotherapy drugs to target multiple cell types within the tumor microenvironment. The potential to target PSCs and the stroma with siRNA nanoparticles will be further discussed below.

Challenges and opportunities in pancreatic cancer

As mentioned throughout this review, one of the most defining and unique features of PC is the highly fibrotic stroma that surrounds and compresses tumor elements [5, 6]. We now have a clear understanding that the stroma is a key player in promoting PC progression, chemoresistance and metastasis [9]. Thus, it is critical to address the role of the stroma when designing new nanotherapeutics for PC. **Figure 3** details key recommendations to be considered when designing and testing nanoparticles for PC treatment.

Stroma-rich solid tumors such as PC generate high levels of solid and fluid stress as well as dense extracellular matrix [83]. Indeed, leaky tumor blood vessels result in increased interstitial fluid pressure (IFP), and this is further exacerbated by the compressed lymphatic vessels that do not allow adequate fluid drainage [84]. Accordingly, tumor IFP becomes comparable to the microvascular pressure thus alleviating the pressure gradient between the blood vessels and the tumor [84]. Remarkably, in a GEMM of PC, IFP was demonstrated to be almost 10-fold higher in PC tumors compared to normal pancreas [10]. As a result, passive diffusion becomes the main transvascular transport mechanism for nanoparticles into the tumor. Given that the rate of diffusion is inversely proportional to the size of the nanoparticle, larger nanoparticles are hindered from diffusing and penetrating solid tumors [85]. Consistent with this research, the majority of successful RNAi-nanoparticles tested in orthotopic PC mouse models are within a size range of 17-60 nm [74, 75, 78, 79]. The importance of nanoparticle size to PC tumor penetration was nicely highlighted in a study by Cabral et al [86], that compared micelle-packaged chemotherapy penetrance into mouse colorectal tumors with penetrance into more fibrotic orthotopic PC tumors in mice. Good accumulation and efficacy was demonstrated in

chemotherapy-loaded micelles ranging in size from 30-100 nm in the colorectal cancer mouse model, whereas only the 30 nm micelles had superior accumulation and anti-tumor effects compared to larger micelles (70 and 100 nm) in the orthotopic PC mouse model [86].

Independent of fluid flow, diffusion of large nanoparticles can also be inhibited by physical interactions with the dense extracellular protein matrix in the stroma [85]. Using computational modeling to understand the transport of gold-nanoparticles through a hydrogel collagen matrix, a recent study demonstrated greater diffusion of smaller nanoparticles (15 nm) compared to larger nanoparticles (100 nm) through the collagen matrix [87]. However, due to the increased diffusion rate, the smaller nanoparticles displayed a greater frequency of collisions with the extracellular matrix proteins compared to the larger nanoparticles [87]. Taken together, these studies clearly illustrate that a fine balance needs to be achieved when determining the optimal size of nanoparticles for stromal rich tumors such as PC. Nonetheless, based on the studies described above, it can at least be hypothesized that nanoparticles within a size ranging from 20-50 nm would be optimal for penetrating PC tumors. However, if the model proposed by Sykes et al [87] is relevant in an *in vivo* setting and is applicable across a broad range of nanoparticle systems, we could predict that increased collisions of smaller nanoparticles with the extracellular matrix might be a useful strategy when designing nanoparticles to selectively target the stroma of PC. This approach can be combined with the addition of targeting moieties specific to PSCs for targeted delivery, as elegantly demonstrated in a recent study [79]. Further studies are warranted to test this hypothesis in clinically relevant and stroma-rich PC models.

In addition to size, the surface charge of nanoparticles needs to be considered when designing them for siRNA delivery to PC tumors. The charge of a nanoparticle can significantly affect its diffusion through a tumor due to potential electrostatic interactions with components of the extracellular protein matrix. Adsorption of serum proteins is also another challenge nanoparticles encounter. Nanoparticles with a high positive surface charge are susceptible to strong binding with a host of different serum proteins including, apolipoproteins, fibronectins and immunoglobulins [88]. The protein corona formed on the surface of nanoparticles can significantly influence their size and charge which can affect cellular uptake [88]. It is accepted that positively charged nanoparticles are more efficient at targeting tumor angiogenic vasculature, compared to neutral or negatively charged particles [89]. This was understood to be the result of irregular and sluggish blood flow that increases the frequency of interactions between positively charged particles and anionic sites on angiogenic tissue of tumor vessels [89]. The protein corona formed on these nanoparticles may have contributed to their altered blood flow. In contrast, another study elegantly demonstrated that neutrally-charged nanoparticles diffuse faster through the extracellular matrix due to less electrostatic interactions with the matrix proteins [90]. Nonetheless, siRNA delivery vehicles often require a slight positive charge to electrostatically bind negatively charged siRNA. Since PC is characterized by a dense network of extracellular matrix in the stroma, it can be concluded that nanoparticles designed for PC should have a close-to-neutral charge.

The size, charge and surface chemistry of nanoparticles can also influence how they interact with other cells in different tissues and can thus affect their toxicity. Nanoparticles must demonstrate a favorable pharmacokinetic profile to maximize their therapeutic potential but must also be adequately cleared from the circulation to limit toxicity [25]. Given the renal system only allows clearance of particles less than 8 nm, most nanoparticles are cleared by the reticuloendothelial and hepatobiliary systems [91]. This involves opsonization where the nanoparticle is coated by nonspecific proteins to allow phagocytosis to occur [92]. The opsonized nanoparticle is then cleared by macrophages in the liver and spleen. Modification of

surface chemistry including PEGylation of nanoparticles can greatly reduce opsonization and phagocytic clearance [93]. There is also evidence that both size and charge of nanoparticles can influence their interaction with the reticuloendothelial system and hence affect their clearance [94-97]. In addition, surface charge can significantly affect the interaction of nanoparticles with the plasma membrane and intracellular components of cells. In general, positively charged particles are believed to be more cytotoxic than neutral or anionic nanoparticles [96]. However, we must remain cautious of applying these general recommendations across all nanoparticle systems, reinforcing the need for robust pharmacokinetic and toxicology *in vivo* analysis of new nanoparticles as part of the pre-clinical development pipeline. In addition to nanoparticle-mediated toxicity, extra considerations need to be made to limit the toxicity and off-target effects of siRNA. However, recent advances in siRNA technology have mostly overcome these challenges by improving the sequence selection and chemical modification of siRNA [98, 99]. The effects of "on-target" silencing of genes in normal non-tumor tissue must also be considered, so emphasis should be placed on selecting genes that are upregulated specifically in tumor tissue. Furthermore, nanomedicine offers the opportunity to actively or passively target tumor cells and can thus overcome this issue.

Overall, all of the above physiochemical properties need to be considered when designing new nanoparticles for PC. In particular, the characteristics of the fibrotic stroma and dense extracellular matrix pose unique drug delivery challenges for PC. However, while the stroma has been classically thought of as a barrier to conventional drug delivery, we propose that the stroma can be considered an opportunity to be harnessed by nanomedicine. In recent years, the concept of stromal reprogramming in PC has become a popular way to improve chemotherapy drug delivery and halt tumor progression. Importantly, nano-based gene-silencing drugs have the potential to silence therapeutic targets specific to PSCs in an attempt to normalise or reprogram the stroma. In fact, there are several different aspects of PSC biology that can be targeted by gene-therapy nano drugs. One aspect of PSC biology that has recently been targeted by an siRNA-nanomedicine used an inorganic gold nanoparticle to deliver HSP47 siRNA to pancreatic tumors *in vivo* [79]. HSP47 is a collagen-specific molecular chaperone involved in the maturation of collagen [100] . As discussed above, knockdown of HSP47 sensitized PC tumors to gemcitabine, a standard of care for PC, which was believed to occur due to a reduction in collagen levels, normalization of tumor vasculature and improved drug delivery [79]. Another approach to reprogram the PC stroma is to inactivate PSCs into their natural quiescent state. PSC quiescence can be induced by blocking the vitamin D receptor which is highly expressed in PSCs. Indeed, treatment with the vitamin D receptor ligand calcipotriol has been shown to reprogram the stroma in a mouse model of PC to improve gemcitabine efficacy [101], and is currently under investigation in clinical trial [102]. There is thus potential here for a nanomedicine-siRNA to be developed to target the vitamin D receptor or other key targets involved in PSC activation. In addition, a recent study elegantly demonstrated that perlecan is a novel therapeutic target intricately involved in the crosstalk between PSCs and cancer cells [103]. Genetic depletion of perlecan in the stroma of PC markedly improved the efficacy of gemcitabine and Abraxane™ in a PC mouse model [103]. There are currently limited pharmacological inhibitors for perlecan, suggesting the potential for a nanomedicine-siRNA approach to therapeutically target this important aspect of PSC biology.

Concluding remarks

The potential to silence any tumor promoting gene with RNAi-nanotherapeutics holds promise for PC treatment. This has been shown by several advanced pre-clinical studies that have demonstrated significant anti-tumor, chemosensitizing and anti-metastatic effects with siRNA-

nanotherapeutics. However, before these promising findings can be translated to the clinic, we need to examine these nanomedicines in pre-clinical models that accurately reflect the biology of human PC. This includes orthotopic PC mouse models with an intact and functional fibrotic stroma, genetically engineered mouse models with a functional immune system as well as complex 3D *in vitro* and *ex vivo* models of the disease. For example, multicellular culture of tumor spheroids in microfluidics devices can recapitulate many microenvironmental cues such as raised IFP, and can thus be a highly useful tool in the pre-clinical development of siRNA-nanotherapeutics [104, 105]. If these models can be designed with high throughput capabilities, nanoparticles with different siRNA targets can be tested on patient tumor samples to guide a personalized medicine program. These models, used in parallel with clinically relevant *in vivo* models, can inform the design of new nanoparticles. We now know that a "one-size-fits-all" approach can no longer be used when designing nanoparticles for cancer treatment. Rather, it is necessary to address the unique pathophysiology of PC, and in particular the fibrotic stroma which presents several barriers for nanoparticle delivery. On the chemistry front, strong consideration needs to be given for developing methodologies that can produce large scale and reproducible amounts of clinical grade nanomaterials. If we are to revolutionize the treatment of PC, it is imperative to build stronger collaborations between chemists, biologists and clinicians, as this will ultimately pave the way for a personalized medicine strategy for PC using siRNA-nanoparticles to inhibit target genes that are unique to a patient's tumor.

Acknowledgements

This work was supported by grants from the National Health and Medical Research Council (NHMRC: P Phillips, J McCarroll and D Goldstein, APP1144108; J McCarroll and P Phillips, APP1144113), Cancer Council NSW (P Phillips, J McCarroll and D Goldstein, RG16-08), Cancer Australia (P Phillips, J McCarroll and D Goldstein, APP1126736), Tour de Cure Project Grants (P Phillips and G Sharbeen; UNSWR002 and UNSWR004), the Avner Pancreatic Cancer Foundation (P Phillips, J McCarroll, D Goldstein and G Sharbeen; APCF0050618) and Cure Cancer Australia Foundation (G Sharbeen, APP1122758). J Kokkinos is supported by an Australian Government Research Training Program Scholarship & UNSW Sydney Scientia PhD Scholarship. G Sharbeen is supported by a Cancer Institute NSW Fellowship (CDF181166). J McCarroll is supported by the Oliva Lambert Foundation.

Author Contributions

JK, RI, EG, JM and PP wrote the manuscript; GS, CB, DG, JM and PP edited and revised the manuscript. APGI provided the human pancreatic tumor specimen in Figure 1.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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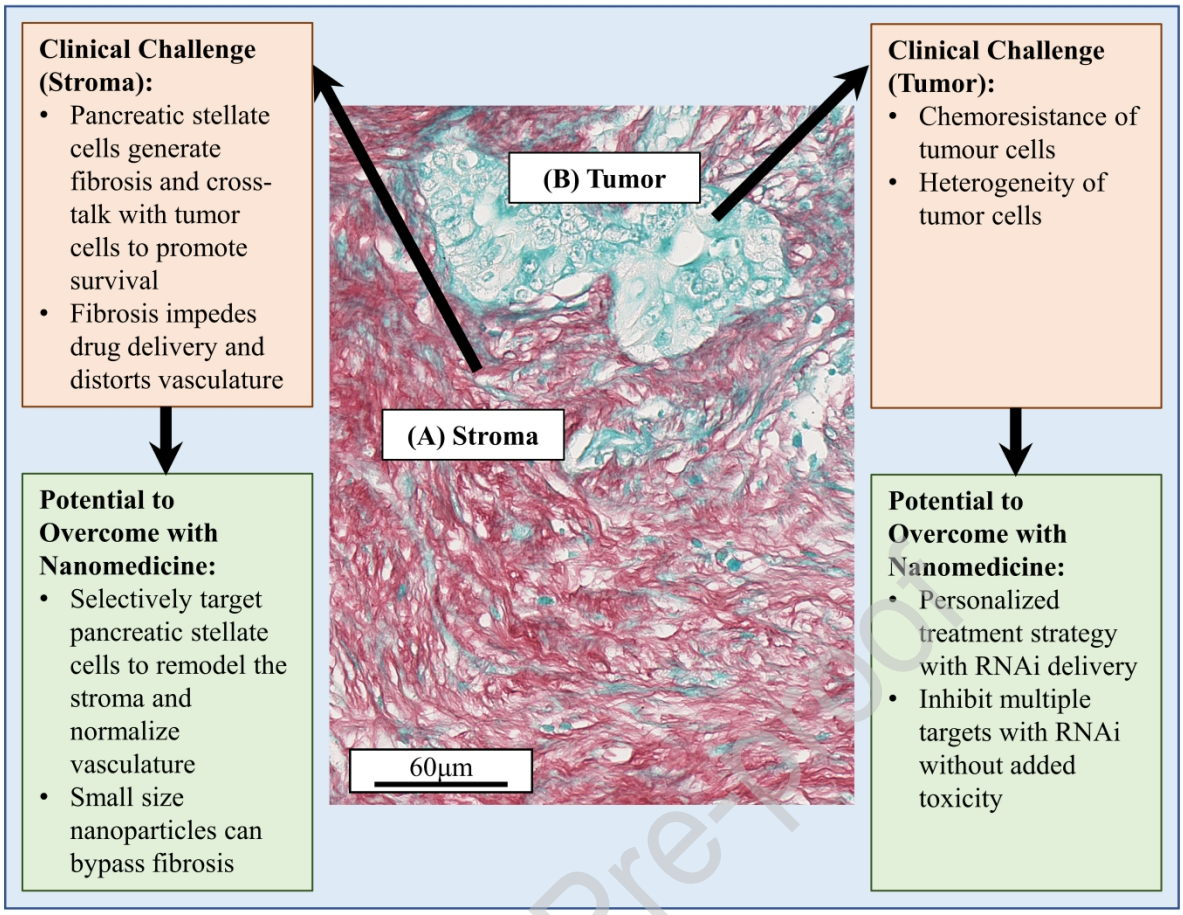

Figure 1. Unique clinical challenges of pancreatic cancer therapeutics give rise to the potential of RNAi-nanoparticles.

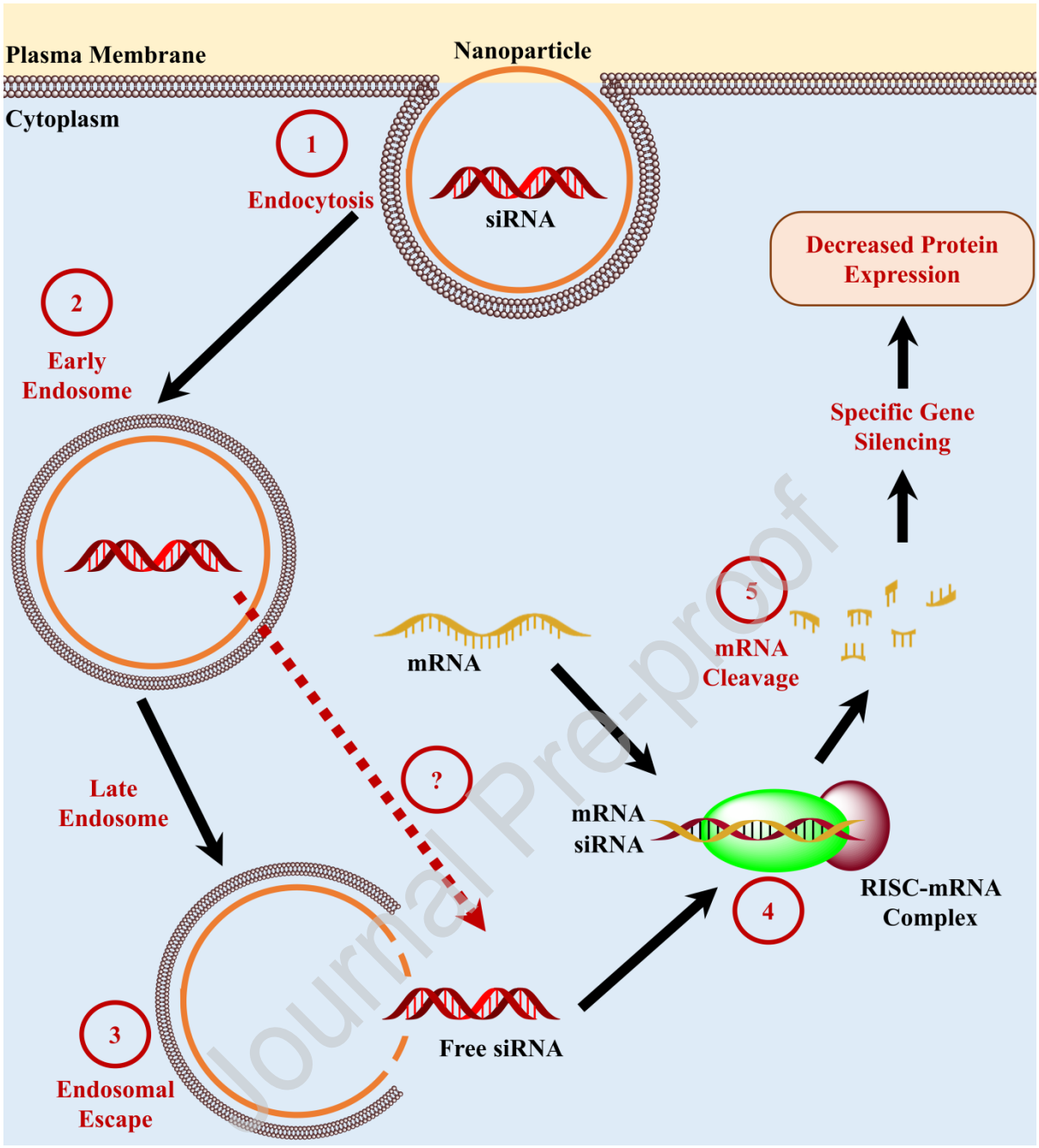

Figure 2. Nanoparticle-siRNA-induced gene silencing.

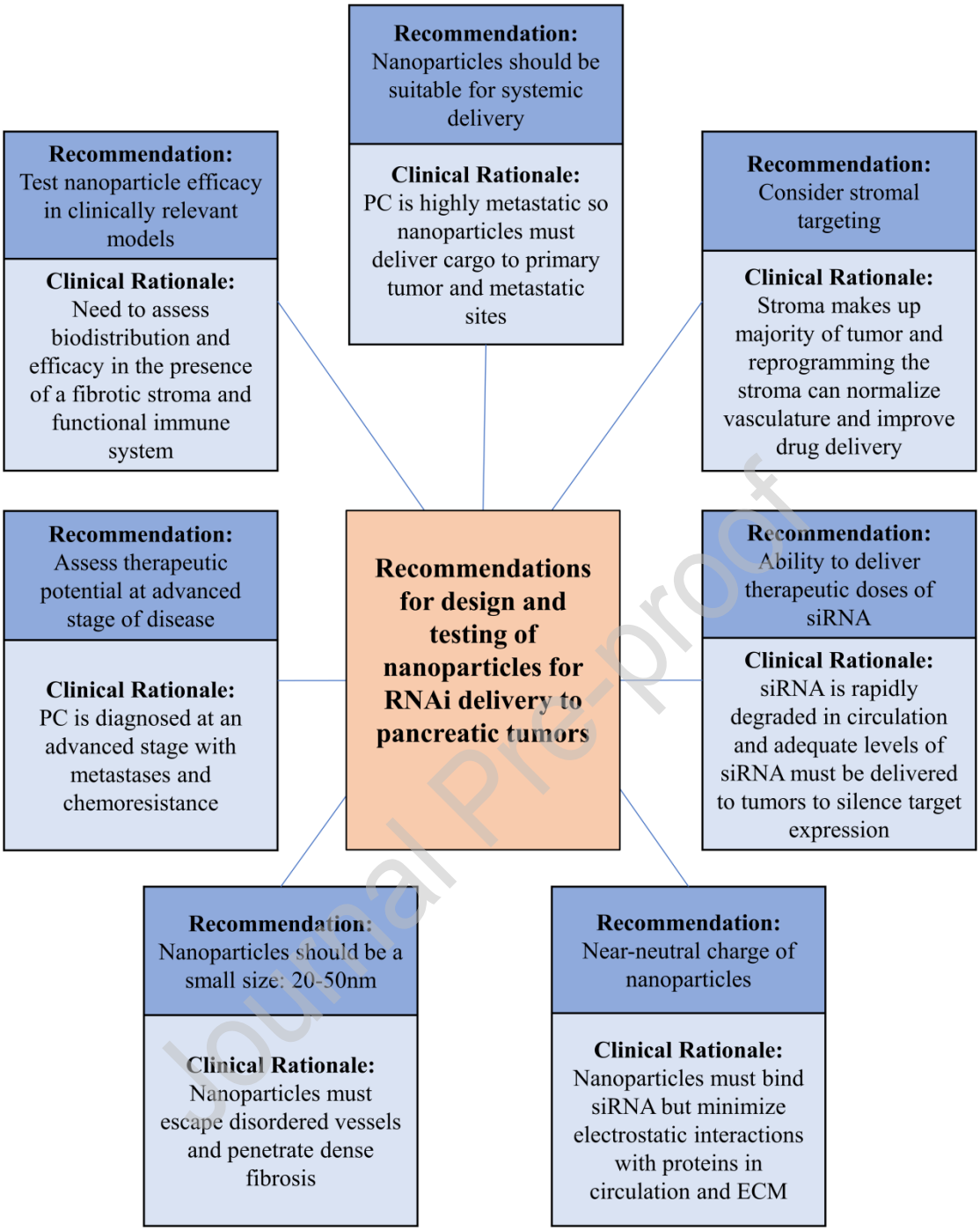

Figure 3. Recommendations for the design and pre-clinical testing of RNAi-nanoparticles in the context of pancreatic cancer.

Figure Legends:

Figure 1. **Unique clinical challenges of pancreatic cancer therapeutics give rise to the potential of RNAi-nanoparticles.** The cross-talk between pancreatic stellate cells and tumor cells promotes the progression of pancreatic cancer, and the potential of RNAi-nanoparticles to specifically target either cell type can inhibit this cross-talk. Furthermore, by selectively targeting pancreatic stellate cells, RNAi-nanoparticles can reduce the fibrosis produced by these cells to normalize tumor vasculature and improve drug delivery. In addition, the heterogeneity and chemoresistance of tumor cells can be overcome by inhibiting multiple targets with RNAi-nanoparticles, as well as the potential for a personalized therapeutic strategy to inhibit genes specific to a patient's tumor. This human pancreatic cancer tissue specimen was collected by surgical removal as part of the Australian Pancreatic Cancer Genome Initiative (APGI) and as approved by the UNSW Human Research Ethics Committee (HC180973). The histological tissue micrograph is a typical human pancreatic adenocarcinoma paraffin-embedded sample stained for Sirius red and methyl green. Sirius red staining for collagen (A) demonstrates the dense fibrotic stroma that surrounds and compresses tumor elements (B), shown in green.

Figure 2. Nanoparticle-siRNA-induced gene silencing. Nanoparticles containing siRNA undergo endocytosis at the plasma membrane to enter the cytoplasm (1). Once in the cell, the nanoparticle-siRNA complexes are trapped in early endosomes (2). There is evidence to suggest that siRNA can escape from early endosomes into the cytoplasm. The early endosomes then mature into late endosomes and fuse with lysosomes, causing the endolysosomal vesicles to swell and eventually burst, releasing the siRNA into the cytoplasm – a process known as endosomal escape (3). The free siRNA is then processed, and a single strand of siRNA binds to the RNA-induced silencing complex (RISC) where it can bind to a complementary mRNA sequence (4). The mRNA sequence is then cleaved into short fragments, thus specifically silencing the target gene which decreases its protein expression (5). **Abbreviations**: siRNA: short interfering RNA.

Figure 3. Recommendations for the design and pre-clinical testing of RNAi-nanoparticles in the context of pancreatic cancer. Abbreviations: ECM: Extracellular matrix; PC: Pancreatic cancer.

Table 1: Current clinical status of RNAi therapeutics (non-cancer).

Disease	Name	Target	Delivery System	Delivery route	Trial Status	Clinical Trial Identifier
METAVIR F3-4	ND-L02-s0201	HSP47	SNALP	I.V.	Phase Ib/II-ongoing	NCT02227459 (2014)
Hypertrophic Scar	STP705	TGF-β1 and Cox-2	Polypeptide NP	Intra-dermal injection	Phase I/II, recruiting	NCT02956317 (2017)
Transthyretin amyloidosis	Patisiran/ ALN-TTR02	TTR	SNALP	I.V.	FDA- and European Commission-approved	NCT01960348 (2018)
CVD, HCL	PRO-040201	ApoB	SNALP	I.V.	Phase I, terminated	NCT00927459 (2009)
CVD, Elevated LDL-C	ALN-PCS02	PCSK9	SNALP	I.V.	*Phase I	NCT01437059 (2011)
Ebola infection	TKM-100201	Viral RNA	Lipid-based NP	I.V.	Phase I, terminated	NCT01518881 (2011)

Abbreviations: METAVIR F3-4, moderate to extensive hepatic fibrosis; CVD, cardiovascular disease; HCL, hypercholesterolemia; LDL-C, low-density lipoprotein-cholesterol; HSP47, heat shock protein 47; TGF- β1, transforming growth factor beta 1; Cox-2, cyclooxygenase-2; TTR, transthyretin; ApoB, apolipoprotein B; PCSK9, pro-protein convertase subtilisin/kexin type 9; SNALP, stable nucleic acid lipid particles; I.V., intravenous; NP, nanoparticle; *, No update posted to date.

Table 2: Current clinical status of RNAi therapeutics (cancer).

Disease	Name	Target	Delivery System	Trial Status	Clinical Trial Identifier	Citations
Neuroendocrine tumors; Adrenocortical carcinoma	TKM 080301	PLK1	SNALP	Phase I/II-ongoing	NCT01262235 (2010)	[106]
Liver, colon, pancreas, gastric, breast, ovarian cancer with hepatic metastasis	TKM 080301	PLK1	SNALP	Phase I-ongoing	NCT01437007 (2011)	[106]
Hepatocellular carcinoma	DCR-MYC	MYC	SNALP	Phase Ib/II, terminated	NCT02314052 (2015)	[107]
Solid and hematological tumors	DCR-MYC	MYC	SNALP	Phase I, terminated	NCT02110563 (2014)	[107]
Solid tumors	CALAA-01	RRM2	Cyclodextrin-containing polymer	Phase I, terminated	NCT00689065 (2008)	[60]
Pancreatic cancer	siG12D LODER	KRAS G12D	LODER polymer	Phase I, completed; Phase II recruiting	NCT01188785 (2010) NCT01676259 (2018)	[108]
Advanced solid tumors; Advanced pancreatic cancer	Atu027	PKN3	SNALP	Phase I and phase Ib/IIa completed	NCT00938574 (2009) NCT01808638 (2016)	[109]
Solid tumors	ALN-VSP02	KSP; VEGF	SNALP	*Phase I	NCT00882180 (2008); NCT01158079 (2010)	[110]
Advanced cancers	siRNA-EphA2-DOPC	EphA2	SNALP	Phase I, recruiting	NCT01591356 (2015)	[111]

Abbreviations: PLK1, polo-like kinase 1; RRM2, ribonucleotide reductase M2; PKN3, protein kinase 3; KSP, kidney specific-cadherin; VEGF, vascular endothelial growth factor; EphA2, ephrin type-A receptor 2; SNALP, stable nucleic acid lipid particles; I.V., intravenous; I.T.; intratumoral; *, No update posted to date.

Table 3: Pre-clinical studies of RNAi nanoparticles in pancreatic cancer.

Delivery vehicle	siRNA target	Size (nm)	PC model	Delivery route	Citations
Liposome	HER-2	100	PANC1 s.c tumors	I.V.	[61]
PEI-complex	CUX-1	-	CAPAN1 s.c tumors	I.T.	[63]
Calcium phosphate polymer	VEGF	100	BxPC3 s.c tumors	I.V.	[62]
PEG-poly-lysine	KRAS	-	PANC1 s.c tumors	I.V.	[64, 65]
PEI-complex	EPAS1	160-22	BxPC3 s.c tumors	NR	[66]
Polylysine co-polymer	HIF-1α	60	PANC1 s.c and orthotopic tumors	I.V.	[74]
Gold nanorods	KRAS	22x47	PANC1 s.c tumors	NR	[70]
PEG-cationic lipoplex	Survivin	225	Hs766T s.c tumors	I.V.	[112]
Superparama-gnetic iron oxide nanoparticles	PLK1	123	Syngeneic orthotopic and GEMM	I.V.	[76]
STAR-PEG	βIII-Tubulin	38	Orthotopic MiaPaCa2 and HPAF-II tumors	I.V.	[75]
PEG-dendrimers	Nur77/ TR3	200	PANC1 s.c tumors	I.V.	[113]
Gold nanoclusters	NGF	17	PANC1 s.c and orthotopic models, and orthotopic PDX	I.V.	[78]
Polyester based vectors	KRAS	100	MiaPaCa2 s.c tumors	P.T.	[114]
Graphene oxide nanoparticles	VEGF	100-250	S180 s.c tumors	I.V.	[115]
Lipid nanoparticle	RRM2	-	PANC1 s.c tumors	I.V.	[116]
Gold nanoparticles	HSP47	41	PANC1/PSC orthotopic tumors	I.V.	[79]
Magnetic nanocarrier	PD-L1	23	Pan02 s.c tumors	I.V.	[72]
Peptide-based nanoparticle	KRAS	55	KPC-1 s.c tumors	I.V.	[117]

Abbreviations: PEI, polyethylenimine; PEG, polyethylene glycol; HER2, human epidermal growth factor receptor 2; CUX-1, cut like homeobox 1; VEGF, vascular endothelial growth factor; EPAS1, endothelial PAS domain-containing protein 1; HIF-1a, hypoxia-inducible

factor 1α; PD-L1, programmed death-ligand 1; PLK-1, polo-like kinase 1; NGF, nerve growth factor RRM2, ribonucleotide reductase M2; HSP47, heat shock protein 47; s.c, subcutaneous; GEMM, genetically engineered mouse model; I.V., intravenous; I.T., intratumoral; NR, not reported; P.T., peritumoral.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: