Evaluation of expansile nanoparticle tumor localization and efficacy in a cancer stem cell-derived model of pancreatic peritoneal carcinomatosis

Aim: To evaluate the tumor localization and efficacy pH-responsive expansile nanoparticles (eNPs) as a drug delivery system for pancreatic peritoneal carcinomatosis (PPC) modeled in nude rats. **Methods & materials:** A Panc-1-cancer stem cell xenograft model of PPC was validated *in vitro* and *in vivo*. Tumor localization was tracked via *in situ* imaging of fluorescent eNPs. Survival of animals treated with paclitaxel-loaded eNPs (PTX-eNPs) was evaluated *in vivo*. **Results:** The Panc-1-cancer stem cell xenograft model recapitulates significant features of PPC. Rhodamine-labeled eNPs demonstrate tumor-specific, dose- and time-dependent localization to macro- and microscopic tumors following intraperitoneal injection. PTX-eNPs are as effective as free PTX in treating established PPC; but, PTX-eNPs result in fewer side effects. **Conclusion:** eNPs are a promising tool for the detection and treatment of PPC.

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Pancreatic peritoneal carcinomatosis (PPC), peritoneal metastasis from primary pancreatic ductal adenocarcinoma (PDAC), remains a significant unresolved therapeutic challenge given its nonresponse to all current therapies and a median survival of 6 weeks from diagnosis [1]. Even for those patients where the tumor is resectable, the 5-year survival rates are 8% for those who do not receive adjuvant chemotherapy after surgery and 21% for those patients who received postresection chemotherapy [2,3]. Moreover, 40–50% of patients who underwent resection of primary PDAC present with PPC [4,5]. Thus, despite advances to date, including improvements to surgery and early detection, PPC remains intractable [1,5–7]. Furthermore, even when PDAC is diagnosed at the precarcinomatosis stage, which is defined by tumor cells in the peritoneal fluid, this has not led to better 5-year survival and is, in fact, associated with a worse prognosis [8,9]. The cur-

rent clinical outcomes for the treatment of PPC demand continued investigation and development of new agents, tumor-specific delivery routes, multicellular *in vitro* models and robust animal models as well as genomic studies to understand the underlying cancer biology in patients. Herein, we address two of these challenges: a nanoparticle system for tumor-specific drug delivery and an *in vivo* pancreatic peritoneal tumor model based on cancer stem cells (CSCs).

The recalcitrance of PPC is attributed, in part, to: the vulnerability of the peritoneum to metastasis and widespread dissemination/progression of peritoneal metastatic tumors or carcinomatosis; increased fibrogenesis/stromal desmoplasia in PDAC tumors; and therapy resistant, tumor-initiating CSCs. Peritoneal susceptibility to metastasis and therapy-resistant tumor-initiating CSCs contribute to the observed rapid-feed forward cycles of dissemination-progression in

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PPC [6,10]. The increased fibrogenesis is thought to act as a physical impediment to tumoral drug delivery, as well as contribute to metastasis [6] while the CSCs are implicated in PDAC recurrence, therapy resistance and metastatic phenotype [11,12]. There are approximately 40,000 cases of PDAC in the USA each year, of which 40–50% develop peritoneal metastasis [4,5]. Moreover, given that bolus intraperitoneal administration of chemotherapy exhibits a short half-life (<12 h) due to peritoneal fluid flux [13,14], it is of importance to develop tumor-localizing drug delivery systems to increase the efficacy of traditional chemotherapeutics. To that end, we recently reported a functional nanoparticle that responds to the lower pH present in tumors to release its cargo through an increase in particle size from 100 to 1000 nm (expansile nanoparticles [eNPs]), and observed that eNPs localize to peritoneal mesothelioma [15,16] and ovarian carcinomatosis [17] warranting its evaluation in another peritoneal malignancy, PPC. eNPs belong to a family of responsive nanomaterials used for drug delivery [18–24].

Another challenge in the study of PPC is the animal modeling of peritoneal carcinomatosis – the widespread dissemination of tumor throughout the peritoneal cavity – that recapitulates associated comorbidities as observed clinically (e.g., ascites, bowel invasion, obstruction and dysfunction, cachexia) and, specifically, the rapid feed forward cycle of tumor seeding, progression and dissemination [6]. Genetically enhanced mouse models, while informative, are logistically difficult due to the complex breeding schemes necessary to attain relevant tumor phenotypes and the inconsistent progression to peritoneal carcinomatosis [25,26]. Similarly, xenograft mouse models, which require administration of 5×10^6 to 1×10^7 non-CSC Panc-1 tumor cells per mouse [25], do not consistently develop peritoneal carcinomatosis and/or, the associated comorbidities of PPC [25], thus decreasing the utility of these modeling system and the applicability of time-course, biodistribution and therapeutic results derived from these models. New models that recapitulate human PPC, its comorbidities and that leverage implantation of anoikis-resistant CSCs and spheroids implicated in metastasis [6,27] are, therefore, needed for the evaluation of novel nano-based delivery platforms for PPC.

In this work, we address these two challenges through an integrative approach. Specifically, we describe: development of a PPC *in vivo* rat model using Panc-1-derived cancer stem cells (Panc-1-CSCs) possessing self-sufficiency in peritoneal tumor seeding, and which recapitulate the comorbidities and rapid progression of human PPC; synthesis of fluorescent, rhodamine-labeled expansile nanoparticles (Rho-

eNPs) and paclitaxel-loaded expansile nanoparticles eNPs (PTX-eNPs) as well as two control formulations; kinetics of *in vitro* cellular uptake of the eNPs in Panc-1 and Panc-1-CSCs; *in vitro* cytotoxicity of PTX and PTX-eNPs against Panc-1 and Panc-1-CSCs; *in vivo* kinetics of Rho-eNP localization to PPC tumors as a function of time after intraperitoneal injection, eNP dose and extent of disease burden; and efficacy of PTX and PTX-eNPs in the newly established *in vivo* Panc-1-CSC-derived model.

Materials & methods

Development of a pancreatic peritoneal carcinomatosis *in vivo* rat model using Panc-1-CSCs

Animal studies were performed in accordance with Boston University School of Medicine (BUSM) Institutional Guidelines. Panc-1-CSCs were isolated as previously described [28]. Briefly, Panc-1 cells in log phase were harvested and subcultured in complete MammoCult® medium (Stem Cell Technologies, BC, CANADA) in 5% CO₂ in a humidified incubator at 37°C. After 2–3 weeks in culture, Panc-1 cells were harvested and plated in complete MammoCult medium containing 0.5% methylcellulose (Stem Cell Technologies, BC, CANADA) in 100 mm ultra-low attachment plates. Peritoneal tumors were developed from septenary CSCs. The Panc-1-CSC-derived peritoneal tumor model was developed in 4–5-week-old nude^{nu/nu} female rats (Charles River Laboratories, MA, USA). The intraperitoneal tumor implantation was performed in anesthetized animals using isoflurane maintained at 1–1.5%. Intraperitoneal injection of 2×10^6 CSCs suspended in 1 ml of M2 media was performed in sterile conditions.

eNP synthesis & characterization

eNPs were synthesized according to published protocols using a two-step sonication-induced mini-emulsion and base-catalyzed polymerization procedure [29,30]. Nonswelling control particles were synthesized with 30% wt/wt cross-linker (30X-eNPs; vs 1% cross-linker in the standard eNP formulation), and generic, non-pH responsive poly(lactic-co-glycolic) acid nanoparticles (PLGA-NPs) were synthesized via mini-emulsion precipitation. The encapsulation efficiency of PTX in PTX-eNPs, PTX-30X-eNPs and PTX-PLGA-NPs was determined by HPLC and consistent with published data [15,30]. eNPs were covalently labeled by copolymerization of a rhodamine methacrylate or an iodinated eNP comonomer, as previously described [15,29]. Particle diameter and zeta potential were characterized via dynamic light scattering (DLS; Brookhaven Instruments Corp., NY, USA) and with a ZetaPALS

zeta potential analyzer by diluting 10 μ l of particles in 3 ml deionized water (~50 kcps).

eNP swelling was monitored according to published procedures [29] by incubating 50 μ l of iodinated eNPs in 2 ml of either 10 mM pH 7.4 phosphate buffer or 10 mM pH 5.0 acetate buffer for 24 h. A 5 μ l drop of the diluted suspension was then adsorbed to a carbon-coated grid that had been made hydrophilic by a 30 s exposure to a glow discharge. Excess liquid was removed with filter paper (Whatman #1) and the samples were stained with 0.75% uranyl formate for 30 s. After removing the excess uranyl formate with a filter paper, the grids were examined in a JEOL 1200EX Transmission electron microscope or a TecnaiG2 Spirit BioTWIN and images were recorded with an AMT 2k CCD camera.

PTX release from each formulation was characterized by diluting 120 μ l of PTX-eNPs, PTX-30X-eNPs or PTX-PLGA-NPs in 14.8 ml of 10 mM pH 5.0 acetate buffer or 10 mM pH 7.4 phosphate buffer with 0.1% wt/wt sodium dodecyl sulfate (SDS) and placing the total volume in 6" of 10,000 molecular weight cut-off snakeskin dialysis tubing. Each bag was suspended in a 1000 ml sink of corresponding composition to the diluting buffer (either 10 mM pH 5.0 or pH 7.4 with 0.1% SDS) and 1 ml aliquots removed at 0, 2, 6, 12 and 24 h, and 2, 4, 6, 8 and 12 days. PTX was extracted from the particles via addition of acetonitrile (1:1, vol/vol) followed by filtration with a 0.22 μ m filter and quantification via HPLC as above.

eNP cellular uptake & cytotoxicity *in vitro*

eNP uptake in Panc-1 cells and Panc-1-CSC spheroids was performed following a published protocol [31,32] by incubation of 25 μ g/ml Rho-eNPs with cells for various durations (0–24 h). At each time point, cells were washed, fixed with 4% formaldehyde and rhodamine accumulation assessed via flow-assisted cell sorting. *In vitro* cytotoxicity assays were performed as previously reported [30,31]; briefly, cells were incubated with treatments (10 μ g/ml–0.1 ng/ml) of PTX-eNPs (1.2 mg PTX/ml; 25 mg polymer/ml), PTX solubilized in 50/50 Cremophor EL/ethanol (CrEL; 1 mg/ml), CrEL without PTX or unloaded-eNPs (25 mg polymer/ml) for 3 days at which point cell viability was assessed via MTS assay (Panc-1) or live-dead staining with propidium iodide (Panc-1-CSCs).

In vivo localization of Rho-eNPs to PPC tumors

Animal studies were performed in accordance with BUSM Institutional Guidelines. Xenograft PPC nude rats were randomly assigned to different study groups. At designated time points after tumor initiation (2 weeks, 5 weeks and end-stage), Rho-eNPs were injected intra-

peritoneally following sterile procedure under isoflurane anesthesia and aseptic preparation of the injection site. Animals received injections of 100 μ L, 300 μ L, or 1 ml of Rho-eNPs (25 mg polymer/ml; 0.2% wt/wt rhodamine/polymer) in the dose-response study and 1 ml in the time-course study. Fluorescent imaging was performed 24 h after Rho-eNP injection unless otherwise specified.

Simulating intraoperative visualization, fluorescent imaging of Rho-eNP localization was performed after midline abdominal incision and retraction under isoflurane anesthesia in a sterile manner using a Woods lamp (long-wave UV), and digital photography was used to document fluorescence. *Ex vivo* fluorescence imaging was also performed using the Woods lamp and digital photography or an Olympus stereo-fluorescence microscope for analysis of cut-surfaces of tumors and normal organs. In the presence of ascites fluid, the fluid was collected and placed into sterile conical tubes for fluorescence imaging with a Woods lamp, and documentation by digital photography. Thereafter, rats were euthanized via removal of vital organs under deep isoflurane anesthesia.

PTX-eNP efficacy against PPC

Thirty-five 4–5-week-old nude^{nu/nu} female rats were injected intraperitoneally with 2×10^6 Panc1-CSCs/rat using the same septenary Panc-1-CSC preparation. Treatments (1 $\times$ /week $\times$ 4 weeks) commenced 2 weeks post cell injections. PTX (10 mg/kg; n = 9), PTX-eNPs (10 mg/kg; n = 9), unloaded eNPs (equivalent polymer dose; n = 9) or saline (n = 8) were administered to PPC tumor-bearing rats. All injections were performed under isoflurane anesthesia and in sterile manner. Rats were monitored daily for health concerns; body weights were obtained weekly; observing monitors were blinded to the treatment group. At the end stage, rats were assessed and euthanized with photo-documentation of ascites, peritoneal tumors and abdominal organ states, and tumor and organ collection for further analysis.

Results

Development of PPC *in vivo* rat model using Panc-1-CSCs

To develop a xenograft tumor model of PPC, we isolated and employed a heterogeneous pool of Panc-1-derived CSCs isolated for anoikis resistance as defined by growth, survival, and spheroid formation in methylcellulose suspension culture through eight passages (Figure 1A). Confocal microscopy of immunostained Panc-1-CSC spheroids revealed the pairwise coexpression of CD133 and CXCR4 – markers of increased metastatic potential [33,34] – and the dual-endothelin1/

VEGF signal peptide receptor, DEspR (Figure 1B).

Following *in vitro* validation of the Panc-1-CSC line, we developed a metastatic PPC xenograft model in nude rats via an intraperitoneal injection of 2×10^6 Panc-1-CSCs, which resulted in rapid tumor establishment throughout the peritoneum. Both large (>1 cm) and small (<0.5 cm) tumors were observed, with larger tumors exhibiting hard nodular features consistent with stromal desmoplasia (Figure 1C). Notably, some rats developed bowel obstruction (Figure 1D), jaundice due to obstruction of the hepatic portal and cachexia (data not shown), as well as ascites (Figure 3 & 4). Histological evaluation of tumors stained with Masson trichrome revealed that even micro tumors (~1 mm) exhibited fibrotic deposition (Figure 1E & F).

The percent tumor take-rate (i.e., the number of animals that develop tumor divided by the total number of animals xenografted) for animals xenografted with 2×10^6 Panc-1 cells was 0%. In contrast, xenograft-

ing with an equivalent number of Panc-1-CSC cells yielded a tumor take-rate of 66% (Figure 1G). By pre-treating animals with cyclophosphamide, the Panc-1-CSC take-rate was improved to >95% and this strategy was used to produce sufficient animals for the ensuing imaging and efficacy studies. In-life, animals displayed comorbidities including ascites, both serous and hemorrhagic, cachexia, with associated gut hypoplasia and decreased activity, gut obstruction, bowel dysfunction and jaundice. At end stage/necropsy, animals displayed retroperitoneal metastases and presented with significant variation in PPC tumor size (Figure 1H).

eNP synthesis & characterization

eNPs (Figure 2A), 30X-eNPs and PLGA-NPs were synthesized according to published protocols with a mean diameter/polydispersity index (PDI) of 150 nm/0.19, 145 nm/0.21 and 210 nm/0.15, respectively, as determined by dynamic light scattering (Figure 2D) [15,29,30].

Figure 1. Panc-1-cancer stem cell-derived metastatic pancreatic peritoneal carcinomatosis xenograft model in nude rats. (A) Panc-1-CSC spheroids isolated based on anoikis resistance in methylcellulose suspension culture through eight passages. (B) Triple immunostaining with markers for CSCs: CD133 (red) [11]; CXCR4 (blue) [11]; and, DEspR (green) [28] – associated with anoikis resistance. Merged signal: magenta-purple: (CD133 + CXCR4), teal (CXCR4 + DEspR), yellow (CD133 + DEspR). (C) Panc-1-CSC peritoneal tumors at end stage with variant sizes consistent with feed forward cycles of tumor progression and reseeding. (D) Large intestine obstruction (white arrow) from tumor invasion (*). (E) Masson trichrome staining of macro- (>0.5 cm) and micro- (<1 mm) tumors. (F) Masson trichrome-stained high magnification of boxed area (□) in Panel E shows collagen deposition (blue) in large tumors >0.5 cm and micro tumors <1 mm in diameter. (G) Comparison of tumor take-rate with different tumor cells (CTX, cyclophosphamide). (H) Tumor volumes of metastatic PPC tumors in a rat xenograft model; end stage reveals a wide range of tumor volumes. CSC: Cancer stem cell; PDAC: Pancreatic ductal adenocarcinoma.

Figure 2. Characterization of PTX-eNP swelling and drug release. (A) eNPs undergo pH-triggered swelling in response to mildly acidic environments found within the microenvironment of large tumors (pH 6.5) or within the late-endosome/lysosome of individual tumor cells (pH 5). (B) Transmission electron micrographs of eNPs maintained at pH 7.4 (left) or pH 5 (right). (C) PTX release from PTX-eNPs, PTX-30X-eNPs and PTX-PLGA-NPs is dose-, time- and formulation-dependent. (D) Table of nanoparticle characteristics for each formulation employed in the drug release study in (C), above. All data are mean ± standard deviation, $n \geq 3$.

eNP: Expansile nanoparticle; PTX: Paclitaxel; PTX-30X-eNP: 30% cross-linked paclitaxel-loaded expansile nanoparticle; PTX-eNP: Paclitaxel-loaded expansile nanoparticle; PTX-PLGA-NP: Paclitaxel-loaded poly(lactic-co-glycolic) acid nanoparticle.

All particles were negatively charged with zeta potentials greater than -40 mV (Figure 2D). The mean encapsulation efficiency of PTX in PTX-eNPs, PTX-30X-eNPs and PTX-PLGA-NPs loaded with 5% PTX (wt drug/wt polymer) was 86, 92 and 78%, respectively, as determined by HPLC. Transmission electron micrographs of eNPs incubated under pH 5 conditions for 24 h revealed an increase in particle diameter and a decrease in particle density (i.e., less contrast/signal) as compared with particles incubated at pH 7.4. Twenty-four hours was chosen as the incubation time for particle swelling studies based upon previously conducted assays [16,29,30] demonstrating observable changes in

particle size and shape within this timeframe. Last, PTX release under sink conditions was pH- and time-dependent for PTX-eNPs, which demonstrated rapid, triggered release at pH 5.0 and slow, eventual leakage at pH 7.4 (Figure 2C). PTX release from heavily cross-linked PTX-30X-eNPs was not pH-dependent and mirrored the slow rate of leakage from PTX-eNPs at pH 7.4. PTX-PLGA-NPs released >80% of their payload within 48 h regardless of pH.

eNP cellular uptake & cytotoxicity *in vitro*

The time-course accumulation of Rho-eNPs in differentiated Panc-1 tumor cells and tumor-initiating

Figure 3. *In vitro* kinetics of cellular uptake and cytotoxicity of paclitaxel-loaded expansile nanoparticles in pancreatic cancer cells (Panc-1) and pancreatic cancer stem cells (Panc-1-CSCs). (A1) In Panc-1 cells, Rho-eNP signal increases rapidly (>80% uptake) within 4 h of incubation at 37°C; (A2) negligible uptake is observed at 4°C. (B1) In Panc-1-CSCs, Rho-eNPs adhere to the surface of cells immediately upon coincubation producing a low-fluorescence ($\leq 10^2$) right 'tailed' distribution at time 0 in approximately 30% of the CSCs. Higher fluorescence intensity and percentage cell uptake are observed at 4 and 24 h. A subpopulation of Panc-1-CSCs (~25–30%) does not demonstrate Rho-eNP uptake/retention even after 4 or 24 h. (B2) Confocal microscopy demonstrates both adherence to the cell surface and internalization of Rho-eNPs in Panc-1-CSCs after 24 h. (C1) PTX-eNPs and PTX demonstrate dose-dependent cytotoxicity against Panc-1 cells and (C2) Panc-1-CSCs. PTX-eNPs exhibit an increased IC_{50} compared with PTX. The vehicle control for PTX (i.e., Cremophor EL/ethanol) shows significant cytotoxicity at concentrations above 0.1% vol/vol while unloaded-eNPs demonstrate negligible cytotoxicity up to concentrations of 125 μ g/ml (data = mean $\pm$ SD, $n \geq 3$). CSC: Cancer stem cell; Panc-1-CSCs: Panc-1-derived cancer stem cells; PTX-eNP: Paclitaxel-loaded expansile nanoparticle; Rho-eNP: Rhodamine-labeled expansile nanoparticle.

Panc-1-CSCs was evaluated by flow cytometry. The time course analysis revealed increasing fluorescence from 1–24 hrs concordant with increasing particle uptake given the same number of fluorescent Panc-1 tumor cells (Figure 3A1, left). Within 4 h of coincubation at 37°C, over 80% of Panc-1 cells demonstrated Rho-eNPs uptake (Figure 3A1, right). This cellular uptake of Rho-eNPs by Panc-1 tumor cells was almost completely inhibited at 4°C (<10% uptake after 24 h coincubation; Figure 3A2) suggesting active, energy-dependent cellular uptake.

In Panc-1-CSCs, which are grown in spheroid clusters, 30% of cells exhibit fluorescence at time 0 (i.e., Rho-eNPs pipetted onto cells and immediately removed followed by washing; Figure 3B1, right), but with lower fluorescence-intensity compared with the 4

and 24 h time points (Figure 3B1, left). Similarly to the increased fluorescence at 4 and 24 h, confocal microscopy revealed a combination of particle internalization and adherence to the cell surface (Figure 3B2).

PTX-eNPs demonstrated dose-dependent cytotoxicity similar to that of free PTX against both Panc-1 cells grown in monolayer (IC_{50} = 5.6 ng/ml, IC_{50} = 2.0 ng/ml, respectively) and Panc-1-CSC spheroids grown in suspension (IC_{50} = 13.8 ng/ml, IC_{50} = 2.1 ng/ml, respectively) (Figure 3C1 & C2). Interestingly, the Cremophor EL/ethanol excipient used to formulate free PTX was found to be significantly cytotoxic at the highest concentrations tested while unloaded-eNPs (vehicle control for PTX-eNPs) exhibited only minimal cytotoxicity at concentrations required to deliver equivalent amounts of PTX.

Time course analysis of Rho-eNP localization to PPC tumors

We evaluated the *in vivo* tumor localization of Rho-eNPs using the Panc-1-CSC-derived human xenograft PPC nude rat model. First, we examined the time-course of distribution of rhodamine fluorescence following injection of a 1 ml dose of Rho-eNPs (25 mg polymer/ml). A hand-held Woods Lamp (long wave UV) enabled straightforward and facile visualization of rhodamine fluorescence within the peritoneal cavity. As shown in Figure 4, time course analysis of identical intraperitoneal doses of Rho-eNPs revealed increasing fluorescence in tumor areas from 1–24 h. Specifically, at the 1 h time point, minimal Rho-eNP accumulation was observed in the tumor with significant fluorescent signal still detected in the ascites and peritoneal fluid (Figure 4A). Increasing Rho-eNP

fluorescence was observed within the tumors up to 4 h and then appeared to stabilize out to 24 h after injection. Rho-eNP fluorescence was observed to increase in proportion to the number of tumors with: greater omentum>gastro-splenic ligament>small intestinal mesentery>retroperitoneal tumors. Notably, in all rats, there was no detectable fluorescence in normal abdominal or pelvic organs (Figure 4), nor in the lungs, heart and brain (data not shown).

Dose-response analysis

Next, we evaluated dose-response effects on localization via intraperitoneal administration of 100 μ l, 300 μ l and 1 ml doses of Rho-eNPs (all 25 mg polymer/ml) with fluorescence-imaging 24 h post injection at euthanasia. As shown in Figure 5, we detected increasing fluorescence within the tumors with increas-

Figure 4. Representative photographs depicting *in vivo* time course of fluorescence after injection of 1 ml of rhodamine-labeled expansile nanoparticle in the peritoneal cavity (n = 2 per time point). (A) 1 h postinjection, the majority of Rho-eNP fluorescence is observed in the ascites while faint accumulation is observed in tumors. (B) 4 h postinjection, the majority of Rho-eNP fluorescence is colocalized with regions of tumor, especially in tumors located in the greater omentum and gastro-splenic (row #1) and small intestinal mesentery (row #2) and, specifically, in microtumors (<1 mm). A visible but faint Rho-eNP signal is observed in the ascites (row #4). (C) 24 h postinjection Rho-eNP fluorescence is only observed in the tumor. No ascites were present and the peritoneal fluid was observed to not be fluorescent.

Rho-eNP: Rhodamine-labeled expansile nanoparticle.

Figure 5. Dose-dependence of intraperitoneal distribution of rhodamine-labeled expansile nanoparticle 24 h post injection ($n = 2$ per time point). (A) 100 μ L injection of Rho-eNPs results in negligible fluorescence in the tumor area in the greater omentum (row #1 and #2). Ascites fluid is not fluorescent, in contrast to Figure 3A (row #4) and B (row #4). (B) 300 μ L injection of Rho-eNPs yields punctate fluorescence colocalized with regions of tumor in the greater omentum (row #1), mesentery (row #2) and lesser omentum (row #3). Ascites fluid is not fluorescent, in contrast to Figure 3A (row #4) and B (row #4). (C) 1 mL injection of Rho-eNPs yields significant tumoral accumulation of fluorescence in all tumor-rich areas. While some macrotumors are fluorescent, fluorescence is detected primarily in microtumors (<1 mm diameter). While no ascites was evident, the peritoneal fluid was also not fluorescent as observed in panels A and B.

Rho-eNP: Rhodamine-labeled expansile nanoparticle.

ing Rho-eNP dose. As with the previous time-course study, Rho-eNP fluorescence was localized to tumor-rich areas including the: greater omentum, lesser omentum and small intestinal mesentery. No fluorescence was observed in normal, abdominal and retroperitoneal organs. Significant particles accumulation was observed in the microtumors.

Rho-eNP localization in early- & late-stage PPC

To determine whether tumor stage/size was a determinant of Rho-eNP localization, we administered a 1 mL injection of Rho-eNPs to animals either 2 (early peritoneal dissemination) or 5 weeks (late-stage disease) postxenografting, and imaged the peritoneum 24 h postinjection. Significant accumulation of Rho-eNPs was observed in earlier-stage tumors with bright signal in many, although not all, microtumors (Figure 6A).

A similar trend was noted at the 5-week tumor stage (Figure 6B). No empirical difference was observed between early- and end-stage accumulation of Rho-eNPs in microtumors. No fluorescence was detected in normal abdominal and retroperitoneal organs or within organs of the reticuloendothelial system (e.g., kidney; Figure 6C, top row). Interestingly, when several large 'macro tumors' (~1 cm diameter) showing fluorescence accumulation were harvested and bisected, Rho-eNPs were found to have penetrated throughout the entire tumor and not just in the superficial layers (Figure 6C, middle row). Furthermore, fluorescence microscopy analysis confirmed tumor-cell localization and sparing of normal mesothelium and tumor microvasculature (Figure 6C, bottom right panel.) Histological evaluation of tissues revealed that Rho-eNPs accumulated both intracellularly and in the extracellular matrix,

thus indicating the potential for intracellular delivery of a drug payload.

PTX-eNP efficacy against PPC

Having observed Panc1 and Panc-1-CSC uptake of eNPs and cytotoxicity from PTX-eNPs *in vitro*, as well as eNP tumor localization *in vivo*, we next determined the efficacy of PTX-eNPs *in vivo*. Beginning 2 weeks postxenografting, rats with established PPC tumors were treated with four weekly 1 ml doses of PTX-eNPs (experimental; 10 mg/kg PTX), PTX (clinical formulation control; 10 mg/kg), unloaded-eNPs (vehicle control) or saline (tumor growth control). Both PTX treatments improved survival compared with the saline and unloaded-eNP control groups (median survival 26 and 29 days, respectively; $p < 0.05$; **Figure 7A**). However, there was no significant difference in overall survival between the PTX-eNP and PTX groups. The study concluded 50 days postxenografting, at which point gross analysis of tumor burden in all remaining animals (average tumor burden scores [0 none, +1 mild, +3 moderate, +5 marked] in three regions: greater omentum, mesentery and retroperitoneum) revealed a trend toward less tumor burden in PTX-eNP-treated animals compared with PTX and unloaded-eNP-

treated animals. However, no statistically significant differences in tumor burden were observed (**Figure 7B**).

Interestingly, while overall survival was not statistically significantly different between PTX-eNP- and PTX-treated rats, their respective in-life activity and degree of cachexia marked by muscle mass and gut size at end stage were observed to be markedly different. PTX-eNP-treated rats were observed to be more active in-life and demonstrated minimal cachexia as confirmed at necropsy; however, the majority of animals displayed significant ascites. The PTX-treated rats were markedly cachectic and exhibited decreased activity and gut hypoplasia at necropsy, while a minority displayed ascites.

The empirical observations of cachexia-associated gut hypoplasia were borne out in histological analysis of gut diameters and wall thickness. To achieve comparable gut segments, analysis was limited to the duodenum within 1 cm of the gastroduodenal junction. Histological analysis of diameters showed smaller duodenal diameters in the PTX-treated group compared with age-matched nontreated controls (**Figure 8A & B**). However, PTX-eNP-treated rats exhibited slightly larger diameters compared with control nontreated nude rats suggesting dilation of the duodenum or

Figure 6. Impact of the amount of disease burden on intraperitoneal distribution of rhodamine-labeled expansile nanoparticle 24 h postinjection. (A) Rho-eNPs colocalize with microscopic disease and medium-sized tumors in early-stage (2 week postxenografting) disease. (B) Rho-eNPs colocalize with microscopic and small tumors in late-stage (5 week postxenografting) disease and exhibit heterogeneous colocalization to larger tumors. (C) In some late-stage tumors, Rho-eNPs adhere to the surface (not shown) while in others, Rho-eNPs penetrate throughout the entire tumor (middle row, scale bar 0.5 cm). No Rho-eNP accumulation is observed in the kidneys (top row). On fluorescence microscopy analysis (bottom row, right), Rho-eNPs (red) are localized in tumor cells with sparing of microvessels (*). Not all tumor cells show Rho-eNP fluorescence and some stromal cells demonstrate Rho-eNP fluorescence (scale bar = 20 μ m); DAPI (blue) nuclear stain. Rho-eNP: Rhodamine-labeled expansile nanoparticle.

Figure 7. In vivo treatment of established, Panc-1-cancer stem cell-derived metastatic pancreatic peritoneal carcinomatosis tumors. (A) PTX-eNPs ($n = 9$) and PTX ($n = 9$) both improve overall survival compared with saline ($n = 8$) and unloaded-eNP controls ($n = 9$). Gehan–Breslow statistic for survival curve, $p < 0.00015$; Holm–Sidak all pairwise multiple comparison test: PTX-eNP versus eNP, $p = 0.026$; PTX-eNP versus saline, $p < 0.007$; PTX-eNP and PTX are not significantly different. **(B)** Gross tumor burden at necropsy. A trend toward less disease burden is seen: tumor burden in control unloaded-eNP > PTX > PTX-eNP rat groups, but differences are not statistically significant. By 42 days after xenografting, all saline-control rats had required euthanasia. eNP: Expansile nanoparticle; PTX: Paclitaxel; PTX-eNP: Paclitaxel-loaded expansile nanoparticle.

less postmortem contraction upon fixation. The latter is likely since analysis of wall thickness reveals that nontreated control duodenal wall thickness is twofold greater than in PTX-eNP-treated animals ($p < 0.01$) (Figure 8B). Notably, duodenal wall thickness in PTX-treated animals is threefold less than controlled nontreated nude rat duodenum.

Discussion

The *in vitro* and *in vivo* model development studies demonstrate the importance of using CSCs to create robust, immunohistologically accurate models of metastatic PPC that recapitulate the heterogeneity and

aggressiveness of this cancer. The mixed expression of multiple CSC-subtype markers (CD133 and CXCR4 – markers of increased tumorigenicity and metastatic potential, respectively [11,27]; and, DEspR – the dual-endothelin1/VEGF signal peptide receptor [28]; The data shown in Figure 1B) are indicative of the heterogeneity of the Panc-1-CSC population and reflects the complex tumor phenotype, which is expected to provide a more robust xenograft model than a single CSC subtype [12]. Additionally, DEspR is associated with anoikis resistance and spheroid formation *in vitro*, and tumor invasiveness and vasculoangiogenesis *in vivo* in subcutaneous xenograft tumors [28]. The simultaneous development of large (>1 cm), small (<0.5 cm) and microscopic (<1 mm) disease reflects the feed-forward cycles of seeding–progression–dissemination in peritoneal carcinomatosis [6]. The fibrotic extracellular matrix (Figure 1E & F) is characteristic of PDAC with the larger tumors associated *a priori* with greater tumor complexity. The heterogeneity in end-stage tumor burden (Figure 1H) is also consistent with repeated feed-forward cycles of tumor seeding, progression and reseeding that occur in metastatic human PPC [5]. Last, the smaller number of Panc-1-CSCs compared with Panc-1 needed to establish xenografts (Figure 1G) is reflective of this aggressive and metastatic characteristic. Altogether, these data validate this model for use in evaluation of the eNP delivery system.

The particle characterization studies demonstrate that eNPs, 30X-eNPs and PLGA-NPs are synthesized with a mean diameter in the range of 100–200 nm and with strong negative surface charges (mean zeta potential >–40 mV for all formulations; Figure 2D). This large range of particle diameters is a result of using a probe-sonicator to generate the miniemulsion during the synthesis procedure; probe-sonicators provide limited control over particle diameter due to rapid dissipation of energy as a function of distance away from the probe tip. As a result of this heterogeneity, eNP tumor localization could not effectively be evaluated as a function of particle size. Studies are ongoing to develop new synthetic methods that utilize microfluidizer technology (Microfluidics, Inc., MA, USA) with the aim of synthesizing relatively monodisperse 50 and 200 nm diameter eNP populations to determine whether these differences in particle size lead to differential particle distribution/tumoral accumulation.

The negative surface charge is a consequence of the anionic surfactant (SDS) coating used to stabilize all three formulations. Surface charge is one of the factors that impacts nanoparticle internalization within cells (in addition to particle size, shape and stiffness), as has been described in a number of papers and reviews [35–39]. We have previously shown that by modifying eNP

Figure 8. Paclitaxel-induced gut atrophy is reduced in paclitaxel-loaded expansile nanoparticle-treated animals compared with paclitaxel-treated animals. (A) Gut wall sections stained with Mason's trichrome (top row, scale bar = 500 μm; bottom row, scale bar = 200 μm). (B) Quantification of mean gut wall diameter (top) and thickness (bottom) (ANOVA with multiple comparison testing * $p < 0.01$; $n = 3$ rats per group with serial sections analyzed from each animal). ANOVA: Analysis of variance.

surface charge and functionality (anionic vs neutral vs antibody [folate]-targeted) the rates of particle internalization can be tuned and, in particular, selectively increased in tumor-cell subtypes that overexpress the folate receptor [40]. However, surface modification can interfere with the pH functionality of the system and, for this reason, it was not employed in the current study [40].

The transmission electron microscopy (TEM) study demonstrates that PTX-eNPs swell in response to a mildly acidic environment (Figure 2B). At pH 7.4, eNPs are solid, dense spheres with well-defined edges. At pH 5.0, the particles increase in diameter and transition to a 'core-corona' state in which a more swollen corona of lower radio-opacity (due to the decrease in polymer density as the particle swells with water) surrounds an as yet unswollen core. These results are consistent with previously published reports showing similar behavior [29].

The drug release study demonstrates pH- and time-dependent release of PTX from PTX-eNPs with <20% release at pH 7.4 and >60% release at pH 5.0 over 24 h (Figure 2C, inset). While this behavior is in accordance with previously published results [30], the current study also demonstrates a previously unreported long-term behavior: PTX eventually leaches out of the particles at pH 7.4 and, after 12 days, >80% of the payload is released regardless of pH. This long-term release study demonstrates that the pH-responsive characteristic is most important to eNP function/PTX release at early

time points. Because particles are observed to accumulate within tumors within 1–4 h of administration (Figure 4), it is likely that PTX release and/or leakage occurs primarily after the particle has reached its target.

Importantly, PTX release from two control formulations: PTX-30X-eNPs – eNPs synthesized with 30% cross-linker to prevent the particle from swelling upon acidification, that is, a 'non-swelling' yet still pH-responsive control; and, PTX-PLGA-NPs – PTX-loaded NPs synthesized from the nonresponsive yet widely employed PLGA polymer, that is, a 'non-responsive' control is strikingly different (Figure 2C). pH-dependent release is not observed from the PTX-30X-eNPs demonstrating that the heavy cross-linking in these eNPs (30 vs 1% in PTX-eNPs) prevents particle swelling and, correspondingly, prevents triggered PTX release. As with the PTX-eNP formulation, PTX eventually leaks from the PTX-30X-eNP formulation over the course of 12 days, regardless of pH. The PTX-PLGA-NP control rapidly releases PTX (i.e., 'burst' release) regardless of pH with >90% released in 48 h demonstrating the importance of the eNP material composition to its functionality as a drug delivery vehicle.

The *in vitro* studies evaluating the uptake of Rho-eNPs into both Panc-1 and Panc-1-CSCs lead to three observations. First, the rapid internalization of Rho-eNPs in Panc-1 cells within 4 h (Figure 3A) is consistent with previous reports in lung, breast, mesotheli-

oma and ovarian human cancer cell lines [17,31,32,41,42]. Furthermore, repeating these studies at 4°C effectively shut down particle uptake, indicating that particle internalization is mediated by energy-dependent endocytic pathways. This is consistent with previous reports detailing eNP internalization in breast and mesothelioma cells primarily via energy-dependent macropinocytosis [17,31]. Interestingly, significant Rho-eNP signal is observed at time '0' for the Panc-1-CSC spheroids. In this study, particles are added to the media, mixed and immediately removed followed by washing and staining of the Panc-1-CSCs. The approximately 30% 'uptake' observed after this short exposure is indicative of the adhesion or sticking of Rho-eNPs to the surface of Panc-1-CSC spheroids consistent with the lower fluorescence detected compared with 4 and 24 h time points (Figure 3B1). Confocal microscopy demonstrates that after 24 h of exposure, some particles are internalized while others remain attached to the Panc-1-CSC spheroid surface or the 3D matrix surrounding the cells; attachment to CSC cell membranes is consistent with a pre-endocytosis stage. This result is in accord with the rapid adsorption of Rho-eNPs to the cell surface/extracellular matrix of the CSC-spheroids leading to the 'immediate uptake' observed via flow cytometry (Figure 3B1). This 'stickiness' of the Panc-1-CSCs, indicated by nonspecific adhesion of Rho-eNPs to spheroids, is consistent with the 'sticky' cellular phenotype observed in U87 glioblastoma spheroids compared with U87-CSC spheroids [28]. As Panc-1-CSCs remain metabolically competent when cultured under cold (4°C) conditions, as is expected of CSCs, [28] the uptake experiments were not performed since reduction in metabolic activity is the primary reason for diminished eNP uptake in 'standard' assay [31].

The results of the *in vitro* cytotoxicity assays are similar to previously published reports of PTX-eNP efficacy against lung, breast, mesothelioma and ovarian cancer. In particular, the trend toward decreased potency of PTX-eNPs compared with PTX, though not significant ($IC_{50} = 1.6$ ng/ml for PTX vs $IC_{50} = 5.6$ ng/ml for PTX-eNPs; $p = 0.60$) is attributed to the 'drug depot' effect wherein the hydrophobic eNP core attenuates the release, and subsequent bioavailability of PTX compared with a bolus treatment of PTX alone [41]. This trend is mirrored in the Panc-1-CSC line with IC_{50} values of 2.1 and 13.8 ng/ml for PTX and PTX-eNP, respectively ($p = 0.30$).

The time-course localization studies demonstrate that the majority of Rho-eNPs localize to tumors within 1–4 h of injection. The presence of Rho-eNPs in the ascites at 1 h and, to a lesser extent, at 4 h is reflective of this time course. The similarity between the 4 and 24 h time points is indicative of the steady-

state nature of the system. The dose-response studies of Rho-eNP fluorescent imaging of PPC tumors showing increasing tumor-associated fluorescence with increasing Rho-eNP doses demonstrate a preference for tumor localization. This result is supported by the consistent sparing of normal mesothelium, abdominal and retroperitoneal organs. Moreover, the nondetection at lower amounts of Rho-eNP also suggests that small injection volumes may not be large enough to bathe all of the tumor bed and, therefore, only a small region of the tumor shows fluorescence; or, the low concentration of Rho-eNPs taken up across multiple micro- and macro-tumors results in particle-tissue concentrations below the visible limit of detection. This suggests that the tumor acts as a 'sink' for Rho-eNPs with a capacity for particle accumulation.

The maximum dose used in this dose-response study was selected to be as large as possible while abiding by Institutional animal care and use guidelines, which are approved for a maximum allowable intraperitoneal injection volume of 1 ml. Beginning with this 1 ml dose, we chose to use two approximately threefold dilutions (300 and 100 μ l) to investigate the impact of decreasing dose as a threefold dilution as this follows typical biochemical dose-response studies wherein threefold dilutions are used for better characterization of the system and equal spacing on a logarithmic scale. Due to the minimal fluorescence observed with the 100 μ l dose, we do not believe further dose titration would provide additional information.

Of particular interest is the differential accumulation of Rho-eNPs between large and small tumors. Rho-eNPs consistently accumulate in small (<5 mm) and micro (<1 mm) tumors; however, particle accumulation in larger, macro (>0.5 cm) tumors is varied and inconsistent. Accumulation in microtumors may be indicative of the increased metabolic rate of these freshly seeded tumor colonies, which results in rapid Rho-eNP uptake. While some macrotumors exhibit significant accumulation and even complete cross-sectional penetration (Figure 6C), others, particularly those with significant stromal desmoplasia, exhibit negligible fluorescence/Rho-eNP accumulation (Figure 4). The mechanism of tumor penetration is not fully understood at this time and further studies are ongoing. Our current hypothesis, based on histopathological analyses documenting the presence of Rho-eNPs in tumor cells (both microtumors and macrotumors) and the simultaneous absence of Rho-eNPs in vascular endothelia, media and microvessels (arterioles, venules and capillaries), as well as Rho-eNP absence in mesothelium (even when immediately overlying tumors) is that Rho-eNPs enter via transmesothelial migration and localize within tumor cells due

to low pH-induced swelling, that is, we hypothesize that tumoral penetration does not occur via circulation through the bloodstream.

At present, based on the localization of Rho-eNP fluorescence in tumor cell clusters, we hypothesize that potential determinants of Rho-eNP localization may include: differences in the degree of tumor fibrosis preventing uptake and intratumoral penetration; preferential localization in tumor cell clusters due to tumor cell metabolic or proliferation states; or, extracellular matrix composition surrounding tumor cells. Further study of *in vivo* mechanisms and correlates of uptake are necessary. Altogether, these observations demonstrate robust Rho-eNP tumor localization, regardless of PPC disease timeline, with a trend toward micro-tumor localization with an optimal dose of 1 ml Rho-eNPs analyzed 24 h after intraperitoneal infusion. Comparative analysis against fluorescent pH(Low) Insertion Peptides (pHLIPs) that have been observed to localize to PDAC and liver metastasis are used for *in vivo* fluorescence imaging to warrant further study, although pHLIPs also accumulated in the kidney [43] whereas Rho-eNPs do not. These results also suggest feasibility for tumor-specific delivery of chemotherapeutic payloads. Imaging of Rho-eNPs with a hand-held UV-lamp (Woods lamp) provides a facile, clinically intraoperative tool for surgeons to detect microtumors without a need for expensive equipment. While the above studies provide a qualitative, as opposed to quantitative, evaluation of Rho-eNP localization, additional studies are under way to develop quantitative measures (e.g., receiver–operator–characteristics curves) to characterize Rho-eNPs tumoral accumulation in peritoneal malignancies, including PPC.

The results from the treatment of established disease model are reflective of the intractable nature of PPC. While both PTX and PTX-eNPs increased overall survival significantly compared with unloaded-eNP and saline controls, all animals either succumbed to their disease or bore significant tumor burden at euthanasia on day 50. This, nevertheless, is significant as the most effective treatment to date for PPC (a multicenter 861-patient cohort, intravenous gemcitabine with nab PTX (Abraxane) [44]) yields a median survival of 8.7 months; an increase of only 2.1 months over gemcitabine alone but with a concurrent increase in grade 3 or higher treatment-related or treatment-emergent adverse events (55 vs 77%, respectively) [38].

While PTX-eNP treatment did not result in improved overall survival over PTX-treated rats, a trend toward less tumor burden, although not statistically significantly different, is observed compared with PTX-treated animals. Importantly, quality of life measures are different between the PTX-eNP and PTX-treated animals. PTX-eNP-treated animals remained

active, curious, and responsive to enrichment stimuli despite some animals bearing ascites. In contrast, PTX-treated rats were overtly cachectic, with reduced activity, noncurious, and nonresponsive to enrichment stimuli. Moreover, at end-stage intra-abdominal inspection, intestinal hypoplasia was evident in PTX-treated rats compared with grossly normal gut morphology in PTX-eNP-treated animals. The reduction in cachexia-associated gut hypoplasia in PTX-eNP-treated rats is borne out in histological analysis of gut diameters and wall thickness. Given the high complication rate with PTX therapy leading to cachexia in 100% of surviving rats, the benefits afforded by PTX-eNPs are encouraging and warrant further study in the future. Comparative analysis with intraperitoneal infusion of gemcitabine [45], other nanoformulations of PTX and combination therapies [38] also warrant further study.

Conclusion

Using Panc-1-derived CSCs, a PPC rat model is described for the evaluation of eNP tumor localization and PTX and PTX-eNP treatment of PPC. eNPs optimally localize to regions of PPC tumor within 4–24 h of injection, with tumor-associated accumulation, which occurs predominantly in microtumors, proportional to the injected dose and independent of tumor stage (early or late). PTX-eNPs demonstrate equivalent performance to PTX alone as normothermic intraperitoneal chemotherapy in a xenograft Panc-1-CSC PPC model, but afford significant improvements in treatment-related toxicity and morbidities, thus presenting a potential novel paradigm to solving the current ‘clinical bottlenecks’ of intraperitoneal chemotherapy. These results substantiate the known difficulty in treating PPC and validate the need for continued research to develop effective and clinically applicable treatments for this intractable disease including new agents, modes of delivery, or targeting strategies.

Future perspective

Nanoparticle-based systems capable of both therapeutic and diagnostic function (i.e., ‘theranostics’) will make inroads into novel treatment paradigms for pancreatic peritoneal metastasis and carcinomatosis, thus opening doors for potentially curative-intent therapy.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- A Panc-1 cancer-stem-cell (Panc-1-CSC)-derived xenograft model of pancreatic peritoneal carcinomatosis (PPC) in nude rats, which recapitulates clinical comorbidities (ascites, cachexia and bowel dysfunction), is reported.
- Panc-1-CSC characteristics, including anoikis resistance, spheroid formation, CSC-associated markers (CD133, CXCR4 and DESP) and increased tumor initiation ability validate the CSC-like nature of this line.
- pH-responsive expansile nanoparticles (eNPs) that swell in response to mildly acidic environments localize to PPC tumors by 4 h of intraperitoneal injection with optimal imaging lasting till 24 h.
- eNP tumor localization is a function of dose and time but is independent of the amount of tumor burden.
- Paclitaxel (PTX)-loaded eNPs are as effective as free PTX in inhibiting Panc-1 and Panc-1-CSC *in vitro*, and treating established PPC *in vivo*; neither treatment prevents tumor progression.
- Intraperitoneal treatment of PPC with PTX-loaded eNPs results in fewer comorbidities than treatment with PTX as determined by in-life behavior, cachexia and postmortem evaluation of gut atrophy.
- PPC remains a significant problem for the oncology community and further effort must be directed toward developing new and more effective therapies for this intractable disease that are better able to harness advantages of intraperitoneal administration for peritoneal carcinomatosis.

References

- Thomassen I, Lemmens VE, Nienhuijs SW, Luyer MD, Klaver YL, De Hingh IH. Incidence, prognosis, and possible treatment strategies of peritoneal carcinomatosis of pancreatic origin: a population-based study. *Pancreas* 42(1), 72–75 (2013).
- Gottlieb S. Resectable pancreatic cancer needs surgery, then chemotherapy. *BMJ* 20, 661 (2004).
- Saif MW. Controversies in the adjuvant treatment of pancreatic adenocarcinoma. *J. Pancreas* 8, 542–552 (2007).
- Warshaw AL, Fernandez-Del Castillo C. Pancreatic carcinoma. *N. Engl. J. Med.* 326(7), 455–465 (1992).
- Lu Z, Wang J, Wientjes MG, Au JL. Intraperitoneal therapy for peritoneal cancer. *Future Oncol.* 6(10), 1625–1641 (2010).
- Sodek KL, Murphy KJ, Brown TJ, Ringuette MJ. Cell–cell and cell–matrix dynamics in intraperitoneal cancer metastasis. *Cancer Metastasis Rev.* 31(1–2), 397–414 (2012).
- Pancreatic cancer treatment – for health professionals (PDQ®). www.cancer.gov
- Vogel I, Kruger U, Marxsen J *et al.* Disseminated tumor cells in pancreatic cancer patients detected by immunocytology: a new prognostic factor. *Clin. Cancer. Res.* 5(3), 593–599 (1999).
- Jayne D. Molecular biology of peritoneal carcinomatosis. *Cancer Treat. Res.* 134 21–33 (2007).
- Tarin D, Price JE, Kettlewell MG, Souter RG, Vass AC, Crossley B. Mechanisms of human tumor metastasis studied in patients with peritoneovenous shunts. *Cancer Res.* 44(8), 3584–3592 (1984).
- Hermann PC, Huber SL, Herrler T *et al.* Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. *Cell Stem Cell* 1(3), 313–323 (2007).
- Penchev VR, Rasheed ZA, Maitra A, Matsui W. Heterogeneity and targeting of pancreatic cancer stem cells. *Clin. Cancer. Res.* 18(16), 4277–4284 (2012).
- Innocenti F, Danesi R, Di Paolo A *et al.* Plasma and tissue disposition of paclitaxel (taxol) after intraperitoneal administration in mice. *Drug Metab. Dispos.* 23(7), 713–717 (1995).
- Tsai M, Lu Z, Wang J, Yeh T-K, Wientjes M, Au J. Effects of carrier on disposition and antitumor activity of intraperitoneal paclitaxel. *Pharm. Res.* 24(9), 1691–1701 (2007).
- Colby A, Liu R, Schulz M, Padera R, Colson Y, Grinstaff M. Two-step delivery: exploiting the partition coefficient concept to increase intratumoral paclitaxel concentrations *in vivo* using responsive nanoparticles. *Sci. Rep.* 6, 18720 (2015).
- Colson YL, Liu R, Southard EB *et al.* The performance of expansile nanoparticles in a murine model of peritoneal carcinomatosis. *Biomaterials* 32, 832–840 (2011).
- Gilmore MD, Schulz MM, Liu MR *et al.* Cytoreductive surgery and intraoperative administration of paclitaxel-loaded expansile nanoparticles delay tumor recurrence in ovarian carcinoma. *Ann. Surg. Oncol.* 20(5), 1684–1693 (2013).
- Colson YL, Grinstaff MW. Biologically responsive polymeric nanoparticles for drug delivery. *Adv. Mater.* 24, 3787–3886 (2012).

- 19 Abouzeid AH, Torchilin VP. The role of cell cycle in the efficiency and activity of cancer nanomedicines. *Expert Opin. Drug Deliv.* 10(6), 775–786 (2013).
- 20 Heidel JD, Davis ME. Clinical developments in nanotechnology for cancer therapy. *Pharm. Res.* 28(2), 187–199 (2011).
- 21 Jolck RI, Feldborg LN, Andersen S, Moghimi SM, Andresen TL. Engineering liposomes and nanoparticles for biological targeting. *Adv. Biochem. Eng. Biotechnol.* 125, 251–280 (2011).
- 22 Langer R. Biomaterials and biotechnology: from the discovery of the first angiogenesis inhibitors to the development of controlled drug delivery systems and the foundation of tissue engineering. *J. Biomed. Mater. Res. A* 101(9), 2449–2455 (2013).
- 23 Traitel T, Goldbart R, Kost J. Smart polymers for responsive drug-delivery systems. *J. Biomater. Sci. Polym. Ed.* 19(6), 755–767 (2008).
- 24 Wang J, Byrne JD, Napier ME, Desimone JM. More effective nanomedicines through particle design. *Small* 7(14), 1919–1931 (2011).
- 25 Day CP, Merlino G, Van Dyke T. Preclinical mouse cancer models: a maze of opportunities and challenges. *Cell* 163(1), 39–53 (2015).
- 26 Ijichi H. Genetically-engineered mouse models for pancreatic cancer: advances and current limitations. *World J. Clin. Oncol.* 2(5), 195–202 (2011).
- 27 Lonardo E, Hermann PC, Heeschen C. Pancreatic cancer stem cells – update and future perspectives. *Mol. Oncol.* 4(5), 431–442 (2010).
- 28 Herrera VL, Decano JL, Tan GA *et al.* DEspR roles in tumor vasculo-angiogenesis, invasiveness, CSC-survival and anoikis resistance: a ‘common receptor coordinator’ paradigm. *PLoS ONE* 9(1), e85821 (2014).
- 29 Colby AH, Colson YL, Grinstaff MW. Microscopy and tunable resistive pulse sensing characterization of the swelling of pH-responsive, polymeric expansile nanoparticles. *Nanoscale* 5(8), 3496–3504 (2013).
- 30 Griset AP, Walpole J, Liu R, Gaffey A, Colson YL, Grinstaff MW. Expansile nanoparticles: synthesis, characterization, and *in vivo* efficacy of an acid-responsive polymeric drug delivery system. *J. Am. Chem. Soc.* 131, 2469–2471 (2009).
- 31 Zubris KA, Liu R, Colby A, Schulz MD, Colson YL, Grinstaff MW. *In vitro* activity of paclitaxel-loaded polymeric expansile nanoparticles in breast cancer cells. *Biomacromolecules* 14(6), 2074–2082 (2013).
- 32 Lei H, Hofferberth SC, Liu R *et al.* Paclitaxel-loaded expansile nanoparticles enhance chemotherapeutic drug delivery in mesothelioma 3-dimensional multicellular spheroids. *J. Thorac. Cardiovasc. Surg.* 149(5), 1417–1425 (2015).
- 33 Bertolini G, D’amico L, Moro M *et al.* Microenvironment-modulated metastatic CD133+/CXCR4+/EpCAM- lung cancer-initiating cells sustain tumor dissemination and correlate with poor prognosis. *Cancer Res.* 75(17), 3636–3649 (2015).
- 34 Zhang SS, Han ZP, Jing YY *et al.* CD133(+)CXCR4(+) colon cancer cells exhibit metastatic potential and predict poor prognosis of patients. *BMC Med.* 10 85 (2012).
- 35 He C, Hu Y, Yin L, Tang C, Yin C. Effects of particle size and surface charge on cellular uptake and biodistribution of polymeric nanoparticles. *Biomaterials* 31(13), 3657–3666 (2010).
- 36 Fröhlich E. The role of surface charge in cellular uptake and cytotoxicity of medical nanoparticles. *Int. J. Nanomed.* 7 5577–5591 (2012).
- 37 Ferrari R, Lupi M, Colombo C, Morbidelli M, D’incalci M, Moscatelli D. Investigation of size, surface charge, PEGylation degree and concentration on the cellular uptake of polymer nanoparticles. *Colloids Surf. B. Biointerfaces* 123 639–647 (2014).
- 38 Morachis JM, Mahmoud EA, Almutairi A. Physical and chemical strategies for therapeutic delivery by using polymeric nanoparticles. *Pharmacol. Rev.* 64(3), 505–519 (2012).
- 39 He C, Hu Y, Yin L, Tang C, Yin C. Effects of particle size and surface charge on cellular uptake and biodistribution of polymeric nanoparticles. *Biomaterials* 31(13), 3657–3666 (2010).
- 40 Stolzoff M, Ekladious I, Colby AH, Colson YL, Porter TM, Grinstaff MW. Synthesis and characterization of hybrid polymer/lipid expansile nanoparticles: imparting surface functionality for targeting and stability. *Biomacromolecules* 16(7), 1958–1966 (2015).
- 41 Zubris KaV, Colson YL, Grinstaff MW. Hydrogels as intracellular depots for drug delivery. *Mol. Pharmaceut.* 9, 196–200 (2012).
- 42 Liu R, Gilmore DM, Zubris KA *et al.* Prevention of nodal metastases in breast cancer following the lymphatic migration of paclitaxel-loaded expansile nanoparticles. *Biomaterials* 34(7), 1810–1819 (2013).
- 43 Cruz-Monserrate Z, Roland CL, Deng D *et al.* Targeting pancreatic ductal adenocarcinoma acidic microenvironment. *Sci. Rep.* 4, 4410 (2014).
- 44 Goldstein D, El-Maraghi RH, Hammel P *et al.* nab-Paclitaxel plus gemcitabine for metastatic pancreatic cancer: long-term survival from a Phase III trial. *J. Natl Cancer Inst.* 107(2), pii: dju413 (2015) (Epub ahead of print).
- 45 Tentes AA, Kyziridis D, Kakolyris S *et al.* Preliminary results of hyperthermic intraperitoneal intraoperative chemotherapy as an adjuvant in resectable pancreatic cancer. *Gastroenterol. Res. Pract.* 2012, 506571 (2012).