Personalized RNA-medicine for pancreatic cancer

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Abstract
Since drug responses vary between patients, it is crucial to develop pre-clinical or co-
clinical strategies that forecast patient response. In this study, we tested whether RNA-based therapeutics were suitable for personalized medicine by using patient-
derived-organoid (PDO) and patient-derived-xenograft (PDX) models. We performed microRNA (miRNA) profiling of PDX samples to determine the status of miRNA deregulation in individual pancreatic ductal adenocarcinoma (PDAC) patients. To deliver personalized RNA-based-therapy targeting oncogenic miRNAs that form part of this common PDAC miRNA over-expression signature, we packaged anti-miR oligonucleotides against one of these miRNAs in tumor-penetrating nanocomplexes (TPN) targeting cell surface proteins on PDAC tumors. As a validation for our pre-
clinical strategy, the therapeutic potential of one of our nano-drugs, TPN-21 was first shown to decrease tumor cell growth and survival in PDO avatars for individual patients, then in their PDX avatars. This general approach appears suitable for co-
clinical validation of personalized RNA medicine and paves the way to prospectively identify patients with eligible miRNA profiles for personalized RNA-based therapy.

Statement of significance: Here we highlight a general approach for personalized RNA-based therapy for PDAC patients. We profiled miRNAs from PDAC patients and packaged an appropriate anti-oncomiR in a tumor targeting nanocomplex. With the example of one of our nano-drugs, TPN-21, on patient avatars (PDO and PDX) we pave the way for personalized RNA-based medicine for PDAC therapy.

Introduction
Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive cancer with a very poor prognosis that progresses rapidly. Multiple therapeutic trials developed to date, such as with chemotherapy, radiation, and anti-stromal therapies have only resulted in small advances, and survival after diagnosis is generally less than a year (1). Clearly, novel therapeutics based on a better understanding of this disease combined with new drug delivery systems are desperately needed. Here we tested a pre-clinical strategy with a novel RNA-based therapeutic tool for personalized PDAC treatment.

MicroRNAs (miRNAs) are a small (~18-25 nucleotides) RNAs that can bind target mRNAs in a sequence-specific fashion to induce their post-transcriptional downregulation. Several studies have already identified miRNAs with significantly altered expression between normal pancreas and PDAC tissues, among them one of the more relevant is miR-21 (2-5). Our lab previously generated a mouse model of miR-21 over-expression and revealed that the mice develop tumors in the tissue where miR-21 is over-expressed, and that the tumors depend on the continued expression of miR-21 for their survival (6). Additionally, our group has already proposed a novel antimiR delivery platform that effectively inhibits oncomiR-155 in another mouse model introducing a new paradigm in the use of antimiRs as anti-cancer drugs (7). These lines of evidence highlight the role of ‘oncomiR addiction’ in maintenance of the tumor phenotype in cancer and open avenues for new therapeutic options (8).

Pancreatic cancer is hypovascularized and largely not penetrable making therapeutic drug delivery a current challenge (9, 10). RNAs are often large and charged, preventing them from easy internalization in the cell. In addition, instability in circulation and rapid renal clearance make the delivery of small RNAs to the tumor site particularly difficult (11). Previous studies testing direct miRNA-based therapy for pancreatic cancer in mouse models have used viral-based vectors, prior antimiR
transfection of tumor cells before inoculation to mouse models or delivery through intratumoral injection (12-16). To date, direct use of antimiRs have failed to target miRNAs in vivo in PDAC (12). We hypothesized that targeted nanoparticles encapsulating oligonucleotide analogues will reduce the rapid clearance of antimiRs and increase their delivery and internalization into the PDAC tumor site to prevent the growth of PDAC tumors. In this new strategy, we used nanoparticles containing "tandem" peptides combining a cell penetrating peptide transportan and tumor penetrating peptide iRGD (17-19) that have been previously shown to deliver siRNAs to the tumor site in a model of ovarian tumor (20). This particle complexes with RNA through charge and hydrophobic interactions to form tumor-penetrating nanocomplexes (TPNs) (21). Since, iRGD has been recently demonstrated to enhance delivery and biodistribution of chemotherapy in PDAC (22), we tested the efficacy of TPNs in the delivery of antimiRs to PDAC.

It is accepted that there is a strong disconnect between pre-clinical work performed in cell lines and xenografts and success of clinical trials (23). For this reason, we tested our miRNA-based therapeutic on patient-derived-xenograft (PDX) samples and patient-derived-organoids (PDO) to better capture human tumor heterogeneity and potentially predict patient response. In our study, through encapsulation in TPN, we successfully delivered antimiR therapeutics to the tumor site for PDAC. We show for the first time, the potential for a new therapeutic precision medicine strategy that monitors levels of oncomiRs in individual patient tumor samples and that tests RNA-based therapeutic nano-drugs in patient avatars to predict future clinical response in pancreatic cancer.

Results
Clinically-relevant oncomiRs form part of a tumor signature in human PDAC patient samples

To identify a PDAC miRNA signature we worked with an initial set of 27 PDAC snap-frozen tumor samples from patient-derived-xenografts (PDX) that maintain tumor architecture and clonal diversity (24, 25). This set has known gemcitabine resistance status (24) and \textit{KRAS} mutation status (\textit{Supplemental Table 1A}). To determine miRNA expression patterns in PDAC tumors we conducted a Firefly™ microRNA Assay of our PDAC set compared to 3 normal pancreatic samples. For these assays, 46 miRNAs already known to be associated with \textit{KRAS}, with pancreatic cancer or similar diseases were pre-selected and profiled from our PDAC samples (\textit{Supplemental Table 1B}). Associated bioinformatics analysis of our profiling revealed the deregulated miRNA expression profile of PDX samples compared to normal samples presented as a heatmap of fold change (\textit{Figure 1A and Supplemental Figure 1A}). An ANOVA test comparing control samples to PDX tumor samples revealed 9 miRNAs significantly deregulated with an adjusted p-value \leq 0.01 (dark dot; miR-217 is out of volcano plot) and additional 4 miRNAs significantly deregulated with raw p-value \leq 0.01 (blue dot) (\textit{Figure 1B}).

To functionally validate the oncomiR signature revealed by the PDAC tumor sample analysis we developed PDAC 3D-models, which formed compact and round spheroids after 7-8 days in culture (\textit{Figure 1C}). We also generated human tumor organoids from 5 independent PDX tumors (Panc 286, Panc 281, Panc 219, PDO 030 and PDO 017) that we characterized and used later as an additional PDAC model for functional tests (\textit{Figure 5 and 6}). Firefly™ microRNA assay was performed on PDAC cell lines, 3D cultures models and PDO to analyze their miRNA signature.
(Supplemental Figure 1B). Interestingly, an ANOVA test comparing all tumor samples (cell lines, 3D-models and Patient-derived-organoid) to control samples (including normal pancreatic cell lines and pancreatic organoid from normal early progenitor) revealed that 6 miRNAs (miR-21-5p, miR-146a-5p, miR-196a-5p, miR-196b-5p, let-7i-5p, miR-29b-5p) from the 13 significantly deregulated miRNAs from PDX profiling are consistently upregulated between PDX and cell line/organoids profiles (Figure 1D). This group of 6 miRNAs forms part of a PDAC signature and reveals a list of potent PDAC oncomiRs that are commonly upregulated in both PDX, cell lines and PDO models. Additional data from a set of 191 patient (Prognostic miRNA database) (26) showed that of these 6 oncomiRs, high miR-21, 196a, 196b and let-7i expression levels in patients are strongly associated with low survival in pancreatic cancer (Figure 1E) and reveal miR-21-5p, miR-196a and miR-196b as a significant potential clinically relevant regulators of PDAC initiation and progression and key miRNAs to detect and target in PDAC using an RNA-based medicine approach.

**OncomiR inhibition limits PDAC 3D-model growth**

In order to test the therapeutic potential of oncomiR inhibition for personalized PDAC therapy, for the remainder of this study, we selected one of the 4 oncomiRs that emerged from our analysis above. We chose to first focus on miR-21-5p for our “proof of concept” study since it has previously been reported to be prognostic in PDAC (27) and because from this set of patient tumor samples, PDAC cell lines and organoids it is part of 8 miRNAs (miR-21-5p, let-7b-5p, let-7a-5p, let-7c-5p miR-22-3p, miR-30b-5p, miR-103-3p and miR-92-3p) found in relative high abundance. First of all, overexpression of miR-21-5p via the Firefly profiling was confirmed in all of the PDAC models by qRT-PCR (Supplemental Figure 2A).
Analysis of each tumor sample’s miR-21-5p expression showed that all the 27 tumor samples upregulated miR-21-5p (Figure 2A), which we confirmed by qRT-PCR (Figure 2B). To support the relevance of this miR-21-5p profile we tested mRNA expression for two PDAC miR-21-5p direct targets, the tumor suppressors programmed cell death 4 (PDCD4) and phosphatase and tensin homolog (PTEN) in these PDX samples (28). PDCD4 and PTEN mRNA expression in tumor samples revealed a significant inverse correlation with miR-21-5p expression for PDCD4 as well as a strong tendency towards downregulation for PTEN (Figure 2C and 2D) supporting a key role played by the oncomiR miR-21-5p in PDAC. To investigate the role of miR-21 in PDAC growth we functionally inhibited miR-21-5p by generating a GFP-stable cell line where miR-21 activity was inactivated via a LentimiRa-Off-hsa-miR-21-5p (Lenti-21). Loss of activity of miR-21 was validated by measuring the rescue of mRNA expression of miR-21 targets including PDCD4 and PTEN (Supplemental Figure 2B) compared to a Lenti-empty vector control.

Lenti-transduced cells were plated to promote spheroid formation as for Figure 1C and their relative growth rate studied. Interestingly, whereas cells transduced by the Lentivirus control (Lenti-control) grew with the same kinetics as un-transfected cells, Lenti-21 spheroids showed a decrease in spheroid number and in size (Figure 2E). Metabolic activity measurements of these 3D-cultures showed that miR-21 inhibition decreased 3D viability by 50%, suggesting that miR-21 inhibition may promote PDAC cell death (Figure 2F). To validate this idea, we stained for apoptosis by using a specific dye that is cleaved in the nucleus under high levels of activated caspase-3. Analysis of the staining showed a significant increase of the caspase-3 activity in the Lenti-21 spheroids at the end of the 9-day assay (Figure 2G).
miR-21-5p has been previously described to promote pancreatic cancer cell invasion by indirectly increasing the mRNA expression of metalloproteinase-2 and 9 (29). We observed that PANC-1 transduced by Lenti-21 expressed a lower level of these two MMP, supporting the PDAC growth delay phenotype in 3D-culture (Figure 2H) (29).

Because it is currently not possible to inhibit miR-21 by transduction of a stable lentiviral construct for human therapy we tested whether miR-21 inhibition by transfection of a mirVana® miRNA inhibitor would show similar effects in PDAC cells. In PANC-1, BxPC3 and PL-45 we obtained respectively, 95.2%, 87.3% and 64.6% decrease in miR-21 expression at the dose of 50nM (Supplemental Figure 2C). The mirVana® miRNA inhibitor was used to inhibit miR-21 in the remaining parts of our study. Through utilization of this anti-miR-21 strategy, we demonstrated that the targeting of relevant oncomiRs counteracts PDAC tumor progression and reveals the potent role of oncomiR inhibition.

Tumor-penetrating nanocomplexes promote potent delivery of RNA-based therapeutics to PDAC

To optimize delivery and targeting of RNA-medicine therapeutic compounds to the tumor site we encapsulated antimiRs in tumor-penetrating nanocomplexes (TPN) that had been described to increase tumor targeting through sequential binding to integrins and NRP1 by tumor penetrating peptide iRGD (20, 22). iRGD specifically binds to αvβ3 integrins, and following proteolytic cleavage, initiates transcytosis and internalization through a semaphorin receptor NRP1 present on tumor cells (17) (30). TPN consists of a C-terminal cell-penetrating peptide iRGD and a N-terminal fatty acid group used to facilitate hydrophobic interactions for self assembly of nanoparticles. Here, consistent with our prior approach we selected anti-miR-21 RNA
that self-associated with and condenses into nanoparticles, called TPN-21 (Figure 3A). As it has already been described that cell penetrating peptide transportan and tumor penetrating peptide iRGD do not bind and penetrate in low integrins-NRP1 pancreatic cells in vitro and in vivo (18, 19, 21), we first validated the expression of the two binding proteins targeted by TPN-21 in PDAC cells by performing Western blots. Semi-quantitative analysis of their expression showed that all the tumor cell lines expressed elevated levels of both receptors (integrins and NRP1), compared to normal pancreatic duct epithelial cell H6c7 (normalized to 1) (Figure 3B). This result indicated that iRGD-containing TPNs should specifically bind integrins/NRP1 positive PDAC tumor models as confirmed Supplemental Figure 3A. Next, we demonstrated the capacity of TPN-21 (100 nM) administration without a transfection agent to inhibit miR-21 expression in PDAC cells at a comparable level to conventional anti-miR-21 transfection (50 nM) (Supplemental Figure 3B).

In order to test TPN in a 3D PDAC model, we first validated the specific binding of the tumor-penetrating peptide (iRGD) in this context. PDAC spheres were incubated with iRGD-TAMRA for 2 hours and observed under a fluorescence microscope. We observed a strong red fluorescent signal on the sphere surface corresponding to iRGD-TAMRA binding after 2-4h incubation (Figure 3C). In addition to surface binding it was important to assay whether the TPNs could be internalized into the sphere. For this purpose, TAMRA-TPN delivery was monitored by confocal microscopy. Z-stack confocal images (Figure 3D) revealed binding and lumen internalization of TPN-21 (TAMRA-TPN-21) into spheres generated with two different PDAC samples.

As previously observed after targeting the stable cell lines to inhibit miR-21 (Figure 2E), repeated administration of TPN-21 (100 nM) following an every-other-
day (QOD) treatment plan (Supplemental Figure 3C) reduced the number and size of 3D-models of PDAC (Figure 3E). Analysis of miR-21 expression by qRT-PCR on treated spheres revealed a 70% knockdown of miR-21, indicating successful delivery and functional activity of the anti-miR-21 compound in the 3D model (Figure 3F). It has already been described that use of tumor penetrating peptide iRGD accumulates in a tumor specific manner in PDAC (18). Here, to validate the *in vivo* homing behavior of iRGD-TPN-21, we generated a PDAC orthotopic model in mice, and performed a single intravenous (I.V) injection of TAMRA-TPN-21 allowing us to assay for TPN-21 fluorescence in mouse organs. Analysis of TPN-21 biodistribution 6 h post-injection was performed by analyzing tissue fluorescence. As commonly found for nucleic acid delivery, after 6 h the highest fluorescent accumulation remains in the liver (31), but relatively high levels of fluorescence was also detected in the tumor site, compared to other organs from the reticuloendothelial system (RES) (spleen and lung) (Figure 3G). All together, these data suggest that iRGD-TPN-21 will promote pancreatic tumor-selective delivery of anti-miR-21 therapy to the PDAC tumor site.

**RNA-based therapy reduces KC model tumor growth and restores PTEN and PDCD4 expression**

In order to explore the clinical potential of the results observed in 3D-models we used a mouse model to test TPN-21 therapy efficiency *in vivo*. We first focused on the D8-175 mouse cell line (mPDAC) derived from *KrasLSL-G12D/+*, *p53fl/fl* (KC) transgenic mice (32) that bear the most common mutations found in PDAC patients (*KRAS* and *TP53*). We demonstrated that mPDAC cells highly expressed the two TPN-21 receptors of TPN (integrins and NRP1) (Figure 3B). We observed an 80-fold upregulation of miR-21-5p by qRT-PCR in mPDAC compared to normal mouse
pancreas (Figure 4A). We also validated that use of anti-miR-21 promotes apoptosis on mPDAC cells by analyzing the co-staining of active-caspase 3 (apoptotic cells) and DAPI (total cells) and observed a 2-fold change in apoptotic rate after anti-miR-21 treatment (Figure 4B).

To test whether use of RNA-based therapy can disrupt tumor maintenance and be a successful approach for PDAC patients we first tested antimiR treatment through TPN-mediated delivery in vivo. We generated a PDAC mouse model by injection of 500,000 mPDAC cells/flank in NOD/SCID mice (Figure 4C). Animals bearing mPDAC allograft were intravenously injected with TPN-control or TPN-21 (5mg/kg of antimiR) 2 times/week over 36 days, starting when the tumor volume reached ~ 150 mm in diameter. Tumor size measurements revealed that delivery of anti-miR-21 significantly reduced mPDAC tumor growth after only 1 injection (Figure 4D). As expected from our 3D analysis (Figure 3E), repeated, systemic administration of the TPN-21 strongly slowed tumor growth along the entire treatment course from day 1 to day 36 and resulted in a 51.1 % suppression of tumor growth (15.07 vs 30.83) relative tumor burden size vs baseline) at the end of treatment (Figure 4E). Interestingly, tumor growth is most affected in the first 24 days of treatment with TPN-21 compared to TPN-control (4.25 vs 14.37 relative tumor burden vs baseline) suggesting a more potent effect of anti-miR-21 in the early stages of tumor growth.

To validate miR-21 targeting to the tumor site following TPN-21 therapy, mPDAC tumors were collected at the end of treatment and miR-21 expression was determined by qPCR. We found that miR-21 expression was significantly decreased in the mPDAC tumors group treated with TPN-21 compared to the mPDAC tumors group treated with TPN-control (Figure 4F). As a consequence of miR-21 inhibition, we observed a significant restoration of miR-21 targets, PTEN and PDCD4 (Figure...
4G). All together, these data showed that use of TPN-21 allowed delivery of anti-miR-21 into the tumor site promoting inhibition of miR-21 expression and upregulation of tumor suppressor PTEN and PDCD4, which correlated with reduced tumor growth. Based on our result we demonstrate that use of TPN approach is suitable for safe and potent delivery of RNA-based therapy (antimiR oligonucleotides) compound to the tumor site.

**TPN-21 impact human PDO growth and predicts PDX response**

To study patient responses to the TPN-21 strategy, we generated human tumor organoids from PDX tumors. We selected one patient sample that has been studied extensively and shows a high degree of concordance between the human sample biopsy and the biopsy growing in mice (24). In addition, this PDX has been characterized as gemcitabine-resistant (Supplemental Table 1B) with a poor therapeutic outcome. To generated PDO 286, fresh PDX tumor fragments were minced and processed accordingly to protocol (33) and grown in matrigel (Figure 5A). These organoids formed as multilayers of cells with diameters between 50-150 µm. They were largely filled with tumor cells, but also exhibited the presence of microlumens. The general morphology of organoids shown by H&E resembled the tumor tissues in vivo (Figure 5B). Additionally, we showed by immunohistochemistry that PDO 286 expressed both TPN receptors (Figure 5B). Before testing our RNA-based strategy on PDO, we first validated that the general miRNA profile was maintained between this PDX and this PDO. By statistically analyzing the miRNA profile of PDX 286 and PDO 286 we observed a general concordance of miRNA expression levels, with strong correlations (Spearman Rho=0.75) and high statistical significance (p-value=2.7e-8) between the two samples (Figure 5C). We also validated that the most deregulated miRNAs from both profiles (miR-21-5p, miR-
146a-5p, miR-196b-5p, let-7i-5p, miR-196a-5p, miR-29b-5p) (Figure 1D) are also commonly upregulated in PDO 286 relative to their own control (Figure 5C).

To investigate the binding and internalization of TAMRA-TPN into the PDO. PDO 286 was incubated with TAMRA-TPN for 2 hours and observed under a fluorescence microscope. We validated that the fluorescent peptide bound 90% of the organoids after 2 hours (Figure 5D). To test TPN-21 on this patient avatar we performed repeated administration of TPN-21 (100 nM) following a QOD treatment plan (Supplemental Figure 3C). We counted the number of organoids growing per well at day 3, 5, 7 and 9 of treatment. A delay in organoid growth was apparent after only 1 treatment, reaching a maximum effect at 9 days (Figure 5E). This observation showed that TPN-21 induced a strong inhibitory effect on tumor growth starting from early times of the treatment course as we previously observed with the mouse cell line in vitro (Figure 4D and 4E). We also verified in this PDO model that miR-21 expression was reduced at the end of treatment (Supplemental Figure 4A). Then, we performed a dose-response study by treating PDO with TPN-21 or TPN-control for 9 days at different concentrations (12.5 nM, 25 nM, 50 nM, 100 nM) following the same QOD treatment plan (Supplemental Figure 3C). We observed that repeated administration of TPN-21 significantly decreased the number of organoids per well from the dose of 25 nM compared to TPN-control, while at the 100 nM dose we observed a decrease of 50% in the number of organoids (Figure 5F). In parallel, we performed a metabolic activity measure by using MTT on the organoids, that confirmed significant inhibition of their viability (42%) after 4 repeated administration of 100 nM TPN-21 (Figure 5F).

To verify that use of the PDO predicts specific patient response to TPN-21 in vivo, we generated a PDX with the cells from Panc-286 PDO to test effects of repeated injection of TPN-21 on PDX tumor growth (Figure 5G). Six-week-old nude
mice were injected with 250,000 cells from PDO 286. When the tumor volumes reached the size of around 100 mm³, xenografts were intravenously injected with TPN-control or TPN-21 (5mg/kg of compound) for a total of 4 injections, using the same schedule applied in the organoid assay. We included a PBS control group to validate that use of the TPN delivery system itself did not significantly impact tumor growth. As observed with PDO 286 (Figure 5E), 4 repeated systemic administrations of TPN-21 strongly slowed tumor growth from day 1 to day 13 and resulted in 54.2% suppression of tumor growth (6.98 PBS vs 6.19 TPN-control vs 3.20 TPN-21 relative tumor burden vs baseline) by the end of the treatments (Figure 5G). Relative tumor burden vs baseline from PBS and TPN-control group are not significantly different at the end of treatment showing that use of TPN itself does not promote anti-tumor effects (Supplemental Figure 4B). All together, these results showed that use of RNA-based oligonucleotides inhibits homogeneously PDO and PDX PDAC growth, highlighting the therapeutic potential of TPN-21 for human PDAC therapy. In addition, here we showed that our approach consisting in utilization of PDAC patient avatars can be used as a rapid pre-screen to systematically investigate patient’s sensitivity to personalized RNA-based therapy.

PDO screen for personalized RNA-based therapy

Since we previously demonstrated that PDO and PDX maintained their general histological characteristics and miRNA profiles (Figure 5B and C) and because PDO can predict PDX responses (Figure 5E-G), we decide to test if we could predict the eligibility for TPN-21 therapy in 4 additional patients through rapid screening of their PDO avatars.

As we are developing TPN-21 strategy (by targeting miR-21-5p among the 4 key candidate miRNAs from our profiling), we started by evaluating the expression
level of miR-21-5p in the 4 PDO at ~ 7 days by qPCR analysis. Among the 4 PDOs studied here, 2 overexpressed miR-21-5p compared to normal organoids, and 2 PDOs did not show overexpression at that stage (Figure 6A). As previously performed in Figure 5, repeated administration of TPN-21 (100 nM) following a QOD treatment plan was performed on these new PDOs (Supplemental Figure 3B). To measure the sensitivity of PDO to TPN-21 we performed a MTT assay to evaluate PDO viability and we measured the size of the organoids at the end of the treatment. We found that only the 2 PDOs overexpressing miR-21 (PDO 286 and PDO 219) showed a significant growth delay after TPN-21 therapy (Figure 6B-E).

In addition to the TPN-21 screen, we treated PDOs with the current standard of care for PDAC patients, gemcitabine. We used gemcitabine at the same dose as TPN-21 for this screen because in 4 cell lines tested for gemcitabine sensitivity, all showed an average IC 50 of 117.25 nM that is consistent with the range used for TPN-21 (data not shown). Here, gemcitabine treatment did not lead to a significant decrease in PDO 286 size or viability in the same dose range as TPN-21 (Figure 6B) whereas PDO 219 was affected. In agreement with previous observations concerning the prevalence of gemcitabine resistance (Supplemental Table 1A), our data suggest the potential benefit of TPN-21 for gemcitabine resistant patients. Consistent with another study combining anti-miR21 and gemcitabine in PDAC, use of TPN-21 therapy sensitized resistant PDOs to gemcitabine treatment by reducing organoids size and viability (Supplemental Figure 5A and 5B) (34).

More interestingly, the 2 “low-miR-21” PDOs (PDO 030 and PDO 017) did not show sensitivity to TPN-21 therapy under these conditions (Figure 6F-I). These results suggest that the patients (PDO/PDX) that do not overexpress miR-21 will potentially not respond to TPN-21 therapy. All together these results demonstrate that a rapid screen of PDO is capable of evaluating and predicting patient response to...
TPN-21 therapy and suggest that miR-21 overexpression in patient samples could be used as a biomarker for predicting favorable outcome to TPN-21 therapy. In addition, our study conducted on PDX 286 and PDO 286 strongly suggest the favorable therapeutically potential of TPN-21 use for gemcitabine-resistant patients.

Discussion

RNA therapeutics for cancer, including anti-miR-21, have been discussed for the past few years but their reported lack of delivery to most disease tissues has limited their therapeutic use to liver and kidney disease (35-37). The work described here demonstrates a targeted approach to deliver RNA-based therapy including antimiRs and potentially mimics, to a specific tumor type (PDAC) or tumor microenvironment where drug delivery is routinely poor. By packaging RNA-based oligonucleotides in tumor-penetrating nanocomplexes (TPN) targeting specific PDAC tumor receptors we propose safe and efficient delivery of RNA therapy, paving the way for the use of TPN-21 as a therapeutic anti-cancer agent for PDAC. We demonstrated that TPN-21 strongly restrains patient-derived-organoid growth, even where gemcitabine failed, and limits PDAC growth in vivo (Figure 5E, F, G and 6B). Currently, numerous, personalized models that help guide precision medicine are emerging (33, 38-40). In this study, through the utilization of PDO as a rapid screen for TPN-21 we show for the first time, the potential of personalized models for RNA-based precision medicine therapy in PDAC. By working with patient avatars we have shown the potential to harness the ability of PDO and PDX models to predict clinical outcomes of patients and thus align pre-clinical work to patient response to TPN-21 therapy, a key goal in personalized medicine (41).
Our work reveals that for PDAC, miR-21 is both a companion diagnostic and the therapeutic target, a theranostic. Upregulation of miR-21 in tumor and stromal tissues is currently clearly associated with several gastrointestinal tumor types (pancreatic cancer, esophageal, gastric cancer, liver cancer), (42, 43) resistance to multiples anti-cancer agents (44) and is a promising non-invasive biomarker for pancreatic cancer risk and detection (24, 45, 46). As miR-21 is already a key detectable biomarker for PDAC (27, 47, 48), in a future Phase I clinical trial, we propose to prospectively identify patients presenting with a similar miRNA signature that could be eligible for this biomarker-based RNA therapy.

In the near future, additional antimiRs (and miRNA mimics) could be packaged into the TPN system and tested on PDO - including the 3 key miRNAs deregulated from our PDAC profiling (TPN-7i, TPN-196a, TPN-196b for anti-let-7i, anti-miR-196a and anti-miR-196b). Use of miRNA-based therapy for PDAC treatment is very novel and is positioned at the forefront of current PDAC and RNA medicine therapeutic challenges. This general approach, described here through this successful proof of principle using anti-miR-21 theranotics, shows very high potential not only for clinical significance but in the establishment of integrative screening methodologies with broad applicability to other cancers and lethal diseases with notoriously dismal clinical outcome.

**Methods**

**MicroRNA profiling**

For miRNA analysis, total RNA was isolated from frozen PDX Tissue samples using the mirVana miRNA isolation kit following standard protocol (Invitrogen). Total RNA samples were sent to Abcam Firefly group for miRNAs Firefly™ Analysis. Data analysis and plotting Heat map and Volcano plots were done through Firefly®
Analysis Workbench software for multiplex miRNA assays. For PDX tissue sample data analysis, miRNAs were normalized using miR-22-3p and miR-30b-5p and were defined by the geNorm-like algorithm. For cell lines, 3D-model and organoids, miRNA were normalized using miR-181b-5p, miR-103-3p, miR-30b-5p which were defined by the geNorm-like algorithm.

**Tumor mouse models**

For generation of PDX mouse models, tumor organoids were grown in 3D tissue culture for 16 days then dissociated with collagenase/dispase, Accutase. Dissociated cells were resuspended in organoid growth media supplemented with 50% Matrigel and 10 uM Y27632 at final concentration 2.5X10^6 cells/ml and the suspension was kept on ice all the time. Six-week old nude mice were injected with 100 µl cell suspension on to the right flank of each mouse. Tumor growth was monitored twice a week by observation and palpation. We defined ~100 mm^3 tumors as the starting point to perform a treatment trial. Mice were treated 2 times a week for a total of 4 intravenous injections with either TPN-21 or TPN-control (5 mg/kg oligonucleotides) or saline solution as an additional control. At the end of the treatment mice were sacrificed, tumors were collected and samples were processed appropriately. All procedures were conducted following an institutionally approved animal IACUC protocol. We conducted therapeutic trials in a subcutaneous model of pancreatic cancer driven by D8-175 cell line. Bilateral flank allografts were implanted on 6-week old NCR/nude mice each seeded with 5x10^5 cells. Mice were divided into groups of 5, with nearly equal average and standard deviation of tumor burden at the start of treatment (~150 mm^3 total tumor burden per mouse). TPN-21 or TPN-control (5 mg/kg oligonucleotides) or saline solution was administrated intravenously twice every week untill the tumor burden reached or exceeded 1 cm according to the
approved animal operation protocol. For biodistribution study, D8-175 cells carrying stably expressed luciferase were suspended in PBS and directly injected into the parenchyma of pancreatic tails of nude mice (5-6 weeks) at 500,000 cells in 100 \( \mu L \) per mouse. The rate of tumor formation was tracked using IVIS system every week after surgical operation. To investigate biodistribution of TPNs in this orthotopic PDAC model, 100 \( \mu L \) of fluorescence labeled nanoparticles (TAMRA-TPN-21) carrying 7.5 \( \mu M \) of miR-21 antagonists were injected through i.v. administration into tumor bearing mice. 4 hours after TAMRA-TPN-21 injection, mice were scarified, major organs (liver, spleen, lung, kidney, heart, tumor) were extracted and scanned using a LI-COR Odyssey Near-Infrared Imaging System. Fluorescence intensity of each organ was measured and quantified using ImageJ.

qPCR

3D-models and organoids were separated from matrigel by washing with PBS. Cell lines, 3D-models and organoids were lysed using trysol and RNA extraction was done using the mirVana miRNA Isolation Kit. cDNA synthesis was performed using miScript II RT Kit (Qiagen) with 250 ng RNA as input. qRT-PCR was performed using the Roche480 Light Cycler ® miRNA system as per manufacturer’s instructions, and gene expression was normalized to U6 and/or 18s. For mRNA analysis, cDNA synthesis was performed using Verso cDNA Synthesis Kit (Life technology) using 500-1000 ng RNA as input. cDNA was used for SYBR Green-based real-time PCR. Gene expression was normalized to GAPDH and or \( \beta \)-Actin. Hsa-miR-21-5p and U6 miScript Primer and 18s, MMP-2, MMP-9, GAPDH, \( \beta \)-Actin, PTEN and PDCD4 QuantiTect Primer are from Qiagen.
Cell lines and antimiR transfection

PANC-1, PL-45, BxPC3, Capan-2 were obtained from the American Type Culture Collection (ATCC), and cultured at 37 °C with 5% CO₂ in DMEM, RPMI1640 and McCoy’s 5a Medium Modified supplemented with 10% FBS and 1% penicillin/streptomycin. H6c7 (Human Pancreatic Duct Epithelial Cell Line). ECA001 were obtained from Kerafast, and maintained in Keratinocyte Basal Medium + supplied supplements (Lonza, Clonetics KBM, Cat#CC-3111). D8-175 is a cell line derived from Pdx1-Cre; Kras^{+/-LSL-G12D}; Trp53 ^{fl/fl} (KPC) mice, and was cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cell lines were tested for mycoplasma using the Lonza MycoAlert™ Detection Kit. For RNA-based functional experiments, mirVana® miRNA inhibitor for hsa-miR-21-5p and mirVana™miRNA Inhibitor, Negative Control #1 (Life Technologies) were used at 50nM and transfected into cells using RNAiMAX (invitrogen), as per manufacture’s protocol.

3D-models and organoids

Snap-frozen PDAC tumor samples used in this work were obtained from the M. Hidalgo’s PDX collection. The pancreatic cancer PDXs were generated as described in (10) and (25). The KRAS mutational status and sensitivity to gemcitabine were routinely analyzed for most of the PDX models within the collection. To generated PDAC 3D-cell culture models, 2000 PANC-1 or BxPC3 cells were mixed with Geltrex® Matrix (Thermofisher) and DMEM or RPMI1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin as 1/1 ratio and plated on non-tissue culture treated 96 well-plates (Falcon, BD NJ, USA). After the matrix solidified, 100 uL medium was added on the well and spheroids were grown.
under standard culture conditions (5% CO₂, at 37°C). Normal human pancreatic progenitors were derived from human pluripotent stem cells through a two-stage induction protocol, in DMEM based media including B27, insulin, hydrocortisone and other factors, as described previously (33). Tumor organoids and PDX mouse models were generated as described by Huang et al (2015). Fresh PDX tumor fragments were minced with a No.22 blade into 1-2 mm small pieces then digested with 1mg/ml collagenase/dispase for 30 minutes, follow by Accutase digestion for 40 minutes. The slurry was re-suspended in DMEM then filtered through a tissue strainer and centrifuged at 1500 rpm for 5 minutes. After aspirating the supernatant we re-suspended the cell pellet with organoid growth medium supplemented with 5% Matrigel and 10 uM Y27632. Organoids were grown under standard culture conditions (5% CO₂, at 37 °C).

To perform a dose-response study, organoids were treated with TPN-21, TPN-control or gemcitabine (HCL, Calbiochem) (12.5 nM, 25 nM, 50 nM, 100 nM) following a QOD treatment plan. Organoids were tested with gemcitabine at 100 nM alone or combined with TPN-21 following a QOD treatment plan.

**Histology and images acquisition**

For histological evaluation, 3D-models and organoids were fixed with 4% PFA and embedded in paraffin. Embedding, Sectioning and Hematoxylin & Eosin were processed by the Histology Core of Beth Israel Deaconess Medical Center. IHC of integrins (Anti-Integrin alpha V antibody (Abcam 179475, 1: 1000)) and NRP1 (anti-NRP-1 (Abcam 81321, 1: 300)) were performed in histology core at the Swanson Biotechnology Center of Koch Institute.
Phase-contrast and Fluorescent images were acquired on an EVOS FL Cell Imaging System with a 4x or 10x or 20x objective. Analysis of number of 3D-models and organoids after TPN-control or TPN-21 treatments was conducted on > 3 independents well per conditions in each experiment for > 3 independents experiments. Confocal images of 3D-models were acquired with the Zeiss LSM 510 Inverted Live-Cell Confocal System using 20x Zeiss Apochromat dry, 0.8 NA at the Confocal Imaging Core of Beth Israel Deaconess Medical Center. All experiments were conducted with > 30 structures evaluated in each experiment.

**Generation of stable cell lines**

To generate the stable pLenti-III-miR-21-off (Lenti-21) line, 2000 PANC-1 cells were seeded in a 96 well plate in DMEM supplemented with 10% FBS. 24 h later, PANC-1 was co-transfected with LentimiRa-Off-hsa-miR-21-5p Virus (abm) or Lenti-III-mir-Off Control Virus (abm) at MOI 5 in the presence of polybrene 8 µg/ul (SIGMA) and Virusplus 1/100 (abm). After 24 h of incubation, medium was replaced with fresh medium for another 24 h before selection of stable GFP positive transductants in media containing 1.5 µg/ml of puromycin for 3 days.

**Viability and apoptotic assay**

Viability assay on 3D-models was performed by using MTT reagents from SIGMA (M2128) according to the manufacturer's recommendation. The apoptosis assay was performed by using NucView™ 530 Caspase-3 Substrate (Biotium). Briefly 3D-models were incubated with NucView reagent (1/500) and Hoescht (1/1000) for 30-60 min and the red signal corresponding to caspase-3 positive structures were counted and normalized to the total number of structures. Viability and apoptosis of 3D-models.
and organoids after treatment was conducted on > 3 independents well per condition in each experiment for > 3 independent experiments.

**Western blotting**

The H6c7 normal pancreatic cell line and PDAC human and mouse cell lines were cultured and lysed in 1x RIPA buffer with protease and phosphatase inhibitor (Thermo Scientific) for 30 min on ice. The whole-cell lysates were then clarified by centrifugation for 25 min at 15,000 rpm at 4°C. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce). Equal amounts (10 μg) of protein samples were fractionated by a Novex® 4–20% Tris-Glycine gel (Thermo Scientific), transferred to nitrocellulose membranes (Thermo Scientific), and blocked in Odyssey blocking buffer (LI-COR Biosciences). Integrin was probed by primary rabbit antibody (1:1000) (Abcam, ab52971), NRP-1 was stained using primary rabbit antibody (1:500) (Abcam, ab81321) and tubulin was measured using primary rabbit antibody (Cell Signaling, #2144). The desired bands were detected by labeling with anti-rabbit (1:10,000) IgG–IRDye 680 secondary antibodies and visualized using the Odyssey Infrared Imaging System (LI-COR Biosciences).

**Tumor penetrating nanocomplex and binding assay**

All peptides in this study are produced by solid phase peptide synthesis CPC Scientific. Tandem peptide (pTP-iRGD) has the sequence CH3(CH)15-GWTLNSAGYLLGKINLKALAALAKKIL-GGK(TAMRA)GGCRGDKGPDC, Cys-Cys bridge was synthesized by CPC Scientific. mirVana™ miRNA inhibitor against hsa-miR-21-5p and non-targeting controls were obtained from Thermo Scientific. PEGylation method of the tandem peptide was previously described (49). To form the
TPNs in solution, miRNA inhibitor, PEGylated pTP-iRGD, and pTP-iRGD were resuspended in nuclease-free water and mixed with the miRNA inhibitor, PEG-containing component, and peptide in 1:2.5:15 molar ratios, first by thoroughly mixing the miRNA with the PEGylated pTP-iRGD and by subsequently mixing in the peptide to create a concentrated solution of TPNs that were adjusted to the desired dilution and buffer composition with appropriate diluent. Binding of iRGD-TAMRA on cells was done at 4 degrees C on ice. The H6c7 normal pancreatic cell line and PDAC human and mouse cell lines (PANC1 and D8-175) were placed for 30 min on ice before addition of iRGD-TAMRA (10 nM). After 15 min treatment with iRGD-TAMRA, cells were washed 3 times with PBS and fixed with 4% PFA and stained with DAPI.

Statistical analysis

Statistical analysis was achieved by using a two-tailed unpaired t test using GraphPad Prism software. 2-way ANOVA have been done for miRNA profiling assays. All graphs show means ± S.D *$P = 0.01–0.05$; **$P = 0.001–0.01$; ***$P < 0.001$; ****$P < 0.0001$ N.S., not significant. For qRT–PCR data, means and s.d. were calculated at the ddCt level before being converted to fold changes as presented in the graphs (50).

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**Conflict of interest:**
The authors declare no conflict of interest.


**Figures legends.**

**Figure 1.**  
A) A heatmap of miRNA expression in PDAC PDX tumor samples (n=27 cases) compared to normal pancreas samples (n=3 control). Normalized miRNA signal intensities are presented as Fold-change (log10 of the ratio between a probe value to the average of all the other samples for that probe). Green represents highly expressed miRNA and red represents lowly expressed miRNA. MiRNA profiling was done through the Firefly Circulating miRNA Assay and normalized using two miRNAs that are not significantly deregulated between all samples, miR-22-3p and miR-30b-5p defined by the geNorm-like algorithm.  
B) Volcano plot of statistical significance against fold-change between tumor samples group and normal samples group, demonstrating the most significantly deregulated miRNAs. Significantly deregulated miRNAs are classified by adjusted p-value (black dot) and raw p-value (blue dot) (≤ 0.01).  
C) Representative phase contrast pictures of PDAC 3D-spheres models from PANC1 and BxPC3 cell lines and PDO Panc 286 and Panc 281. Scale bars, 1000μm  
D) Overview of the top commonly deregulated miRNAs from PDX and cell line/organoid profiling. The list of miRNAs was generated by matching the 14 miRNAs significantly deregulated (≤ 0.01) from PDX profiling with the 14 miRNAs most deregulated (ranked by p-value) from cell line-organoid profiling. miR-21-5p* is the only miRNA significantly upregulated in both profiling experiments: (+) is used for
upregulated miRNAs and (-) for downregulated miRNAs. **E** PROGmiR representation of miR-21-5p, miR-146a-5p, miR-196b-5p, let-7i-5p, miR-196a-5p and miR-29b-5p expression and overall survival in pancreatic adenocarcinoma. High miR-21-5p, miR-196b-5p, let-7i-5p, and miR-196a-5p expression are clinically correlated with decreased overall survival in pancreatic cancer.

\* P = 0.01–0.05; ** P = 0.001–0.01; *** P < 0.001; **** P < 0.0001 N.S., not significant, two-tailed \*t\*-test.

**Figure 2. A)** Relative expression (mean of fluorescence) of miR-21-5p in the set of PDAC tumors compared to normal controls. **B)** qPCR analysis of miR-21-5p expression level in the set of PDAC tumors compared to normal controls with a dynamic range from 3 to 22 fold. **C and D)** qPCR analysis of PTEN and PDCD4 expression levels compared to normal controls. **E)** Growth kinetics of Lenti-21 3D-model cells from day 1 to 9. Quantification of the number of organoids per well at the end of treatments. Scale bars, 400μm. **F)** Cell metabolic activity measures from MTT assay at 9 days of 3D-model growth and associated representative pictures. Scale bars, 1000μm. **G)** NucView™staining (red) report significant increase in caspase-3 activity in Lenti-21 spheroids. Quantification of caspase-3 positive spheroids. **H)** qPCR of MMP-2 and MMP-9 expression level in PANC1 stable cell line (Lenti-21). For qPCR each miRNA samples were normalized on the basis of both its U6 content and on the basis of GAPDH for mRNAs. Scale bars, 50 μm Error bars, mean ± s.d \* P = 0.01–0.05; ** P = 0.001–0.01; *** P < 0.001; **** P < 0.0001 N.S., not significant, two-tailed \*t\*-test; n = 3 biological replicates.
Figure 3. A) Representation of tumor-penetrating nanocomplexes (TPN) loaded with anti-miR-21 (TPN-21). B) Western blot analysis of two receptors, (NRP1 and integrins) bound by the TPN. Column 1 (H6c7 normal pancreatic cell lines), columns 2-7 (PDAC human and mouse cell lines). Western blot band corresponding to NRP1 and integrins have been quantified against tubulin levels and normalized against normal cell lines (1). C) TAMRA-TPN-21 (red) binding in PDAC 3D-models after 1h of treatment. Nuclei were stained with DAPI. PANC-1 Scale bars 100μm, BxPC-3 Scale bars, 50μm. D) Z-stack Confocal images show binding of TAMRA-TPN-21 to the PANC1 and BxPC3 spheroid and internalization into the lumen after 2h treatment. E) Quantification of number and size of 3D-sphere after TPN-21 treatment and representative picture of Bxpc3 and PANC1 3D-models after repeated TPN-21 treatments. Spheres size was measure through imageJ software on a total of > 35 spheroids and are presented in arbitrary units (UA). F) qPCR analysis of miR-21-5p expression level after TPN-21 treatment.

G) Tumor biodistribution of fluorescent-TPN-21 in organs (1:Liver, 2:spleen, 3:lung, 4:kidney, 5:heart) and tumor site (6: pancreas) after 48h injection in an orthotopic KC model of PDAC (n=2). For qPCR each miRNA sample was normalized on the basis of its 18s content and on the basis of GAPDH for mRNAs.

Error bars, mean ± s.d *P = 0.01–0.05; **P = 0.001–0.01; ***P < 0.001; ****P < 0.0001 N.S., not significant, two-tailed t-test; n = 3 biological replicates and n = 2 biological replicates for Western Blot.

Figure 4. A) qRT-PCR of miR-21-5p expression level in the PDAC (mPDAC) cell line compared to normal pancreas. B) Nucview staining (red) normalized to total cells stained with DAPI (blue) report significant increase of the caspase-3 activity after TPN-21 treatment in mPDAC cells. C) mPDAC flank tumor model. D) Relative tumor
burden after (1, 3, 5 and 10 I.V injection of TPN-control n=7 or TPN-21 n=10 (5mg/kg).

**E** Relative tumor burden after I.V injection of TPN-control or TPN-21 (5mg/kg) over the 36 days. **F** qPCR analysis of miR-21-5p expression level in the tumor site at the end of treatment (TPN-control n=5; TPN-21 n=5). **G** qPCR analysis of PTEN and PDCD4 expression level at the end of treatment (TPN-control n=5; TPN-21 n=5). For qPCR each miRNA sample was normalized on the basis of its 18s content and on the basis of beta-actin for mRNAs.

Error bars, mean ± s.d *P = 0.01–0.05; **P = 0.001–0.01; ***P < 0.001; ****P < 0.0001 N.S., not significant, two-tailed t-test; n = 3 biological replicates.

**Figure 5.** **A** Representation of PDO 286 and PDX-286 model. **B** Representative H&E pictures of PDX-286 and PDO 286. Immunohistochemistry staining for two tumor-penetrating receptors, (NRP1 and integrins) on PDO 286. **C** Overview of miRNA expression profile of PDO 286 and PDX-286. The list of miRNAs was generated by considering only the 6 miRNAs highly deregulated from both profiling experiments (Figure 2B). These 6 miRNAs are commonly upregulated (+) in both PDX and PDO, relative to their own control (normal pancreas for PDX profiling) and (normal organoid for PDO profiling). **D** TAMRA-TPN-21 binding in PDO 286 after 1h of treatment. Nuclei were stained with DAPI. TAMRA-TPN-21 is red. Scale bars 400μm. **E** Number and size of PDO 286 after treatment by TPN-21 or TPN-control (1, 2, 3 and 4 treatments) at the optimum dose of 100nm. Dose-response curve of PDO 286 treated with TPN-21 or TPN-control compared to untreated organoids. (Doses are 0, 12.5nM, 25nM, 50nM, 100nm). **F** Cell metabolic activity measured from MTT assay at 9 days of 3D-culture growth. **G** Representation PDX-286 generation from PDO 286. Tumor size of the PDX after I.V injection of PBS (n=6), TPN-control (n=6) or TPN-21 (n=5) (5mg/kg) over the 13 days treatment and representative pictures of
PDX tumors after treatments with PBS, TPN-control or TPN-21. *P = 0.01–0.05; **P = 0.001–0.01; ***P < 0.001; ****P < 0.0001 N.S., not significant, two-tailed t-test.

**Figure 6. A)** Representative pictures and qPCR analysis of miR-21-5p expression levels in 4 PDO (Panc 286, Panc 219, PDO 030 and PDO 017.) Scale bars 1000μm. 
**B,D,F and H)** PDO metabolic activity measured from MTT assay after repeated TPN-21 treatments and representatives pictures of PDO 286, PDO 219, PDO 030 and PDO 017. Scale bars 1000μm. **C, E, G and I)** Quantification of organoid size after TPN-21 treatment. Organoids size was measure through ImageJ software on a total of ≥ 35 spheroids and are presented in arbitrary units (UA). For qPCR each miRNA sample was normalized on the basis of its 18s content. *P = 0.01–0.05; **P = 0.001–0.01; ***P < 0.001; ****P < 0.0001 N.S., not significant, two-tailed t-test.
Figure 1

A

B

Volcano for group

1 probe

Log10 (Significance)

Log10 (Fold-change)

C

Patient-derived xenograft

Cell lines and organoids

D

miR-217-5p
-miR-148a-5p
-miR-139a-3p
-miR-148b-5p
-miR-222-5p

E

3 years 5 years

miR-21-5p

miR-146a-5p

miR-196b-5p

let-7i-5p

miR-196a-5p

miR-29b-5p

Overall survival

p-val: 8e-04

p-val: 0.5981

p-val: 0.0077

p-val: 0.573

p-val: 0.0157

p-val: 9e-04

3 years 5 years

3 years 5 years

3 years 5 years

High Expression

Low Expression

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Figure 2

A. miR-21-5p MFI

B. miR-21-5p relative expression

C. PDCD4 relative expression

D. PTEN relative expression

E. Lenti-control and Lenti-21 organoids

F. Growth inhibition

G. Caspase-3 positive sphere

H. MMP2 and MMP9 relative expression
Figure 3

A

Tandem peptide + miRNA Antagonist anti-miR-21 + PEGylated tandem peptide → Tumor Penetrating Nanocomplex (TPN)

C

PANC1 BxPC3

DAPI/TAMRA-TPN-21

D

PANC1 BxPC3

E

E.

PANC-1

Number of organoids (% of control)

0 20 40 60 80 100 120

TPN-control TPN-21

Sphere area (UA)

0 2000 4000 6000 8000 10000 12000

TPN-control TPN-21

Bxpc-3

Number of organoids (% of control)

0 20 40 60 80 100 120

TPN-control TPN-21

Sphere area (UA)

0 2000 4000 6000 8000 10000 12000

TPN-control TPN-21

F

miR-21 relative expression

0.0 0.2 0.4 0.6 0.8 1.0 1.2

TPN-control TPN-21

miR-21 relative expression

0.0 0.2 0.4 0.6 0.8 1.0 1.2

TPN-control TPN-21

G

TAMRA-TPN-21

Relative fluorescent units

1 2 3 4/5 6

liver spleen lung kidney heart tumor
Figure 5
Figure 6

A

B

C

D

E

F

G

H

I

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