Title

Hyaluronic acid nanoparticle-encapsulated microRNA-125b repolarizes tumor-associated macrophages in pancreatic cancer.

Journal

Nanomedicine (London, England), 16(25)

ISSN

1743-5889

Authors

Parayath, Neha N
Hong, Brian V
Mackenzie, Gerardo G
et al.

Publication Date

2021-10-01

DOI

10.2217/nnm-2021-0080

Peer reviewed

ABSTRACT

Aims: The goal of this study was to investigate a novel strategy to target tumor-associated macrophages and reprogram them to antitumor phenotype in pancreatic adenocarcinoma.

Methods: We conjugate M2 peptide to HA-PEG/HA-PEI polymer to form self-assembled nanoparticles with miR-125b. LSL-KrasG12D/+, LSL-Trp53R172H/+, Pdx1-Cre genetically engineered mice model of pancreatic ductal adenocarcinoma was used to evaluate efficacy of HA-PEI/PEG-M2Peptide nanoparticles.

Results: We demonstrate in-vitro M2 macrophage specific delivery of targeted nanoformulations. Intraperitoneal administration of M2-targeted nanoparticles showed preferential accumulation in the pancreas of KPC-PDAC mice and a >4-fold increase in the M1-to-M2 macrophage ratio compared to transfection with scrambled miR.

Conclusions: M2-targeted HA-PEI nanoparticles with miR 125b can transfect TAMs in pancreatic tissues and have significant implications in PDAC immunotherapy.

Keywords: tumor-associated macrophages, microRNA transfection, hyaluronic acid-poly(ethylene imine) nanoparticles, intraperitoneal administration, KPC mice, pancreatic adenocarcinoma.

Summary Points

- In this study we have successfully conjugated M2 peptide to HA-PEG polymer.
- M2 peptide conjugated to HA-PEG polymer with HA-PEI polymer in 1:1 ratio can self-assemble in nanoformulations in presence of positively charged micro-RNA.

- The synthesized HA-PEI/PEG M2 Peptide nanoparticles can specifically target M2 macrophages in vitro and reprogram them to anti-tumoral M1 phenotype.

- In LSL-KrasG12D/+, LSL-Trp53R172H/+, Pdx1-Cre (KPC) genetically engineered mice model of pancreatic ductal adenocarcinoma (PDAC), intraperitoneal administration of HA-PEI/PEG M2 Peptide nanoparticles results in accumulation of HA-PEI/PEG M2 Peptide nanoparticles in pancreatic tumors

- Additionally, intraperitoneal administration of HA-PEI/PEG M2 Peptide nanoparticles can reprogram M2 TAMs to anti-tumoral M1 phenotype in tumor microenvironment.

1. INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a devastating disease [1]. Surgery, which offers the only realistic hope, has a limited role, with less than 20% of patients undergoing successful resection that affects the clinical outcomes. A significant hurdle in PDAC treatment stems from the presence of a complex tumor microenvironment, consisting of a stromal shell rich in fibroblasts, endothelial cells, and the tumor core, consisting of tumor cells and infiltrated tumor-associated macrophages (TAMs). Generally, macrophages are highly plastic cells, playing a crucial role in the innate and adaptive immune responses to pathogens and inflammatory stimuli. They can be activated and polarized to two distinct phenotypes: the classically activated (M1) and alternatively activated (M2) macrophages [2, 3].

Among the cells associated with the tumor microenvironment, TAMs are important regulators modulating multiple steps in tumor development including immune suppression, tumor angiogenesis, tumor growth, metastasis, and chemoresistance [4]. During PDAC progression, TAMs switch to M2-like phenotype increasing fibroblastic morphology, upregulating mesenchymal markers, promoting proliferation and migration [5]. Not surprisingly, M2-polarized TAMs infiltration in pancreatic cancer samples has a negative correlation with prognosis [5, 6]. Therefore, repolarization of TAM's from a predominant protumor (M2) to

antitumor (M1) phenotype appears to be a promising strategy for the treatment of aggressive tumors, such as PDAC.

Previous studies have indicated that microRNAs (miRs) in the tumor microenvironment can affect TAMs' phenotype. Thus, the evaluation of molecular/cellular mechanisms of how miRs modulate TAM polarization is an area of intense investigation. In particular, miR-125b, which is expressed in macrophages than in other immune cells at high levels, can regulate macrophage activation [7]. However, delivery of nucleic acid poses significant challenges, such as poor *in vivo* half-life, cell penetration incapability, and instability within the cell endosomes. Among various delivery systems, non-viral vectors have been embraced for gene delivery applications primarily due to their safety, versatility and ease of production, application, and scale-up ability [8]. Hyaluronic acid (HA) polymers are rich in hydroxyl and carboxyl functional groups that can be exploited for chemical derivatization with different modalities (**Figure 1**) and can specifically binds to CD44 receptors overexpressed in macrophages [9,10]. The efficacy of HA-PEI nanoformulations encapsulating miR-125b to reprogram the TAMs to anti-tumor (M1) phenotype and in-turn resulting in anti-cancer efficacy have been previously demonstrated [10-13].

Moreover, for M2 macrophage-specific targeting, Cieslewicz, *et al.,* recently reported of a peptide, called M2pep, with the sequence of YEQDPWGVKWWY, which preferentially bind to M2 macrophages upon systemic delivery [14]. The M2pep conjugated to a pro-apoptotic peptide shows preferential binding, rapid internalization and accumulation in murine TAM's compared to other cells [14]. In this study, we used the M2pep's targeting capability and prepared HA-M2pep conjugates for incorporation in HA nano-assemblies (**Figure 1**). Therefore, our innovative delivery system enables two levels of targeting; HA polymer will target the CD44 receptors on the macrophage cell surface, while the M2pep peptide will allow specific targeting of TAMs in the tumor microenvironment. A hallmark of pancreatic cancer is its inability to respond to immunotherapy, with nearly all PDAC patients failing to benefit from current immunotherapeutic drugs. Thus, there is an urgent need to explore new strategies, including those targeting TAMs, to improve the response to immunotherapy. The aim of this proof-of-concept study is to further enhance the targeting ability of HA-PEI nanoparticles to specifically M2 macrophages in vivo.

In this study, we have developed a novel microRNA (miR)-based therapeutic paradigm to reprogram TAMs from a pro-tumor M2 to an anti-tumor M1 phenotype in the clinically relevant *LSL-Kras*$^{G12D/+,}$ *LSL-Trp53*$^{R172H/+,}$ *Pdx1-Cre* (KPC) genetically engineered mouse model of pancreatic cancer. It is noteworthy that KPC mice, which conditionally express endogenous mutant Kras and p53 alleles in pancreatic cells [15], develop pancreatic tumors as they age, and recapitulate pathophysiological and molecular features that resemble those observed in human pancreatic ductal adenocarcinoma [16]. We showed effective delivery, transfection, and TAM repolarization with miR-125b administered with M2 peptide conjugated HA-PEI/HA-PEG NPs in vitro and in vivo.

2. MATERIALS AND METHODS

2.1 Materials

Sodium hyaluronate (HA) 20 – 40K was purchased from Lifecore Biomedical Co. (Chaska, MN). Branched poly(ethyleneimine) (bPEI) 10,000 Da was purchased from Polysciences Inc. (Warrington, PA). N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS). Mono-functional poly(ethylene glycol)-amine (PEG2K-NH2, molecular weight 2,000 Da) was purchased from Creative PEG Works (Winston Salem, NC). Dialysis membrane of 12-14 kDa molecular weight cut-off (MWCO) was obtained from Spectrum Laboratories Inc., CA. Recombinant murine interleukin 4 (IL4) was obtained from PeproTech (Rocky Hill, NJ). Cy5-NHS ester was obtained from Lumiprobe (Hallandale Beach, FL). Primers specific for iNOS-2, Arg-1, and β-actin were purchased from Eurofins MWG Operon (Huntsville, AL). The M2 peptide or scrambled peptide with a spacer sequence of GGGC was synthesized at Tufts University Core Facility, Boston, MA

2.2 Cells

J774A.1 adherent murine macrophage cell line obtained from the American Type Culture Collection (ATCC; Manassas, VA) was cultured in Dulbecco's modified Eagle medium (DMEM) (Cellgro, Manassas, VA) containing 10% fetal bovine serum (FBS) (HyClone, Logan, UT) at 37 °C and 5% CO_2.

2.3 Synthesis of HA-PEI and HA-PEG-M2 conjugates

HA-PEI was synthesized as previously described [12,13]. For the synthesis of HA-PEG-M2 50 mg of maleimide-PEG-amine was added to EDC/NHS-activated HA. Following synthesis of HA-PEG-maleimide, an M2 peptide, YEQDPWGVKWWY or scrambled peptide was used for conjugation with maleimide. The carboxyl group of terminal cysteine of the peptide was reacted with the maleimide of maleimide-PEG-HA in HEPES buffer (pH 7.4) at 1:1 molar ratio while mixing under N2 at 4 °C for 24 hours. The peptide conjugate was then purified by dialysis (3.5 kDa). The purified polymers (HA-PEI, HA-PEG-M2and HA-PEG-scrambled peptide) were lyophilized using a freeze dryer for 48 h (Freezone-6, Labconco Inc., Kansas, MO) and the conjugation was confirmed by 400 MHz 1H-NMR spectroscopy (Varian Inc., CA). Cy5-NHS ester and nanoparticles were dissolved in PBS at 10:1 ratio for overnight reaction. Next day, they were dialysed overnight with 12-14 kDa MWCO membrane in de-ionized water. The conjugate was then lyophilized for 48h and then stored at -20°C. Cy5 grafting on polymer was measured by in-direct method by quantifying Cy5 in dialysates using Synergy H1 microplate reader (BioTek, Vermont, US).

2.4 Formulation and characterization of HA-PEI/HA-PEG-M2 nanoparticles

HA-PEI/PEG and miR125b were mixed in 54:1 weight ratio to form the HA-PEI/HA-PEG-M2 nano-systems. Briefly, HA-PEI and HA-PEG-M2 were dissolved in PBS (pH 7.4) at 3mg/ml in 1:1 ratio to which miR was added at 0.5mg/ml and stirred for 20 mins at RT. The percent encapsulation efficiency (%EE) was measured using RiboGreen® assay kit as per manufactures instructions. Dynamic light Scattering (DLS) (Zetasizer, Malvern Analytical) was used to measure particle size, size distribution and zeta-potential.

2.5 Uptake of HA-PEI/HA-PEG-M2 nanoparticles by J774 macrophages

J774 murine macrophages cells were seeded on coverslips or in a 6-well plate for 2 h at 37 °C with 5% CO2. Cells were then supplemented with conditioned media (IL-4, 100 ng/ml) for 6 h, followed by treatment with Cy5 labeled HA-PEI/HA-PEG-Scram peptide or HA-PEI/HA-PEG-M2 peptide nanoparticles for 2 h, 4 h, 6 h and 12 h. Cells on coverslips were then fixed with 4%

paraformaldehyde (PFA) for 10 min at RT. Using the mounting media containing DAPI, the coverslips were then mounted on the slide and imaged (excitation wavelength 647nm) using Zeiss confocal microscope (Carl Zeiss, Cambridge, UK). For FACS analysis using Multi-Laser Flow Cytometry (DXP11 Analyzer), the cells in 6-well plate were fixed fixed with 4% PFA for 10 min at RT before analysis.

2.6 *In vitro* polarization of J774 macrophages using miR125b encapsulated HA-PEI/HA-PEG-peptide

The ability of HA-PEI/PEG-miRNA nanoparticles to modulate macrophage phenotype from M2 to M1 was assessed by measuring the change in the expression level of iNOS (M1 marker gene) and Arg-1 (M2 marker gene) in J774A.1 macrophages after transfection with the nanoparticles. J774 murine macrophages cells were seeded in a 6-well plate for 2 h at 37 °C with 5% CO2. Cells were then supplemented with conditioned media (IL-4, 100 ng/ml) for 6 h, followed by treatment with 100 nM miR125b encapsulated HA-PEI/HA-PEG-Scram peptide or HA-PEI/HA-PEG-M2 peptide nanoparticles. The cells were harvested at 24 hours after the initial addition of the nanoparticles for RNA isolation using a Zymo RNA Isolation kit. cDNA synthesis was performed using Verso cDNA Synthesis kit from Thermo Scientific Inc. (Waltham, MA). The polarization effect was determined by quantifying the expression of iNOS and Arg-1 using qPCR (LightCycler 480, Roche, Branford, CT). qPCR results were normalized to the housekeeping geneβ-actin, and data was analyzed using Prism 6 software.

2.7 Polarization of macrophages using miR125b encapsulated HA-PEI/HA-PEG-peptide in LSL-Kras$^{G12D/+,}$ LSL-Trp53$^{R172H/+,}$ Pdx1-Cre (KPC) mice

The first in vivo study involved assessing the presence of HA-PEI-Cy5-M2 pep nanoparticles in the pancreas of KPC mice. For this purpose, 12-week KPC mice were administered with HA-PEI-Cy5-M2 peptide or HA-PEI Cy5-Scramble peptide (50 mg/kg) by intraperitoneal injections. At 2 h post administration, all mice were euthanized, and pancreas, lungs, liver, kidney, brain, heart, and spleen were collected. All organs were imaged using the IVIS system (Perkin Elmer, Hopkinton, MA) and the intensity of the Cy5 signal was determined and compared between the groups.

Next, we evaluated the effect of intraperitoneal administration of HA-PEI-miR-125b in repolarization of TAMs in KPC mice bearing pancreatic tumors, by assessing the phenotype of TAMs following administration of HA-PEI-miR-125b conjugated with M2 peptide. For this purpose, KPC mice bearing tumors, were injected intraperitoneally (2x/week for 2 weeks) with HA-PEI- miR-125b NPs conjugated with M2 peptide or HA-PEI- scramble miRNA NPs conjugated with M2 peptide. We evaluated the expression levels of iNOS and Arg-1 (M1 and M2 markers, respectively) in the macrophage population isolated from the pancreatic tumor tissue by qPCR, and the expression levels of Arg1 staining by IHC. At the end of the 2-week treatment, pancreas/tumors were either fixed with 4% PFA overnight at 4ºC for immunohistochemistry or homogenized for isolation of macrophages. The embedded pancreas/tumor tissue was sectioned (5 μm), rehydrated, and stained using standard immunohistochemistry protocols using F4/80 (Cat # 70076) and Arg1 (Cat # 93668) antibodies (Cell Signaling Technologies, Danvers, MA), as previously described [17, 18]. Random five sections from each tissue section were quantified using ImageJ software.

TAMs were isolated by digesting pancreatic tumor tissue in DMEM media with 2mg/ml of collagenase A (Roche) and 13 DNase I (Sigma) for 30 min at 37°C on a shaker. The reaction was halted with 5ml of fetal bovine serum (FBS) (Atlanta Biologicals) and cells were collected by filtering through 40 mm Nylon mesh and centrifugation at 2,000 rpm for 5 min at 4°C. The collected cells were then resuspended in staining buffer (PBS containing 1% BSA). MACS magnetic selection using anti-mouse F4/80 microbeads (Miltenyi Biotec, San Diego, CA) was used for isolating F4/80+ cells. High Pure RNA isolation kit (Roche Applied Sc, Indianapolis, IN) was used to isolate RNA from F4/80+ cells and converted to cDNA using capacity cDNA Reverse Transcriptase (Applied Biosystems, Grand Island, NY). Primers against iNOS, Arg-1, CD86, CD80, CD206, CD163 and Beta-actin (housekeeping) genes were used for expression levels of the respective genes using qPCR (iCycler, Bio-Rad, Hercules, CA).

2.8 Statistical Analysis

Statistical analysis was performed using Prism 7.0 software (Graph Pad Software, San Diego, CA). The data, obtained from at least three independent experiments, were expressed as the *mean* ± *SD*. Statistical evaluation was performed by one-factor analysis of variance

(ANOVA) followed by the Tukey test for multiple comparisons. $P<0.05$ was regarded as statistically significant.

3. RESULTS

3.1 Formulation and characterization of HA-PEI/HA-PEG-M2pep nanoparticles encapsulating miR-125b

The HA-PEG polymer was successfully conjugated to M2/Scrambled peptide **(Figure 1A)**. The ^{1}H-NMR spectra of the peptide show the characteristic aromatic ring peaks between 6.5 -7.5 ppm (**Figure 1B**). The 1H-NMR spectra of the HA-PEG-Peptide also shows the characteristic aromatic ring peaks between 6.5 -7.5 ppm. The HA-PEI conjugation strategy and method we used in this study is same as in our previous study [12]. The 1H NMR spectrum of the HA-PEI with EDC/NHS coupling does not show the PEI peak but shows additional peaks between 2 and 3 ppm due to PEI conjugation on the HA backbone. During the self-assembly process, the PEI forms the positive core that electrostatically binds miR, while the HA forms the negatively charged surface of these nanoparticles (**Figure 1C**). The encapsulation of miR, resulted in similar sizes with M2 (216±9 nm) and scrambled peptide (210±12 nm) conjugated polymer as determined by the DLS (**Figure 1D**) and our previous studies with TEM (Supplementary Figure 1) images [12]. The zeta-potential on HA-PEI/HA-PEG/HA-M2pep particles was -18±4 mV and HA-PEI/HA-PEG/HA-scrambled peptide particles showed was -21±5 mV **(Figure 1D)**. The %EE of miR-125b in HA-PEI nanoparticles as well as the miR-negative control was 99% as shown by the RiboGreen assay. Furthermore, based on the start ratios of polymer:miR and the %EE, we can deduce that the ratio polymer and miR-125 was maintained at 54:1 post-formulation process.

3.2 *In vitro* uptake of HA-PEI/HA-PEG-M2pep nanoparticles encapsulating miR-125b by J774 macrophages

To demonstrate that HA-PEI/HA-PEG-M2pep nanoparticles can be taken up by tumor associated macrophages, the nanoparticles were labeled with Cy5 dye. J774 murine macrophages were treated with IL-4 (100 ng/ml) for 6 h prior to treatment with HA-PEI/HA-PEG/HA-M2pep

particles to develop the tumor-associated M2 phenotype. After IL-4 treatment, the cells were treated with Cy5 labeled HA-PEI/HA-PEG/HA-M2pep or HA-PEI/HA-PEG/HA-scram peptide for 2, 4, 6 and 12 h. As determined by confocal microscopy **(Figure 2A)** and FACS analysis **(Figure 2B)**, it was observed that HA-PEI/HA-PEG/HA-M2pep nanoparticles show a higher uptake as compared to HA-PEI/HA-PEG/HA-scram peptide nanoparticles at 4 and 6 h. At 12 h there was a gradual decrease of fluorescence for both the formulations.

3.3 *In vitro* repolarization of M2 macrophages using HA-PEI/HA-PEG-M2pep nanoparticles encapsulating miR-125b

Once the successful uptake HA-PEI/HA-PEG/HA-M2pep nanoparticles by the M2 macrophages, we next tested the efficacy of HA-PEI/HA-PEG/HA-M2pep nanoparticles loaded with miR-125b in macrophage repolarization from M2 to M1 in vitro. For this purpose, the M2 phenotype was induced in the J774 macrophages by treatment with IL-4. As shown in **Figure 3**, IL-4 treatment significant reduced iNOS levels and significant increased Arg-1 levels, demonstrating M2 polarization of the macrophages. Following IL-4 administration, J774 macrophages were treated with HA-PEI/HA-PEG/HA-M2pep nanoparticles encapsulated with miR-125b. The cells treated with HA-PEI/HA-PEG/HA-M2pep nanoparticles and HA-PEI/HA-PEG/HA-scram pep nanoparticles showed increase in Arg-1 levels compared to untreated control. However, comparing the HA-PEI/HA-PEG-M2 pep group with HA-PEI/HA-PEG-scram pep, we show that HA-PEI/HA-PEG-M2 pep can reduce Arg1 levels as compared to HA-PEI/HA-PEG-scram pep treated group when cells are pre-treated with IL4 (mimicking anti-inflammatory microenvironment). Additionally, a significantly higher level of iNOS were observed in cells treated with HA-PEI/HA-PEG/HA-M2pep nanoparticles as compared to cells treated with HA-PEI/HA-PEG/HA-scram pep nanoparticles. Additionally, a significant increase in iNOS/Arg-1 levels (~100-fold) was observed in HA-PEI/HA-PEG/HA-M2pep nanoparticles treated group as compared to HA-PEI/HA-PEG/HA-Scram pep nanoparticles **(Figure 3)**, clearly demonstrating the enhanced ability of HA-PEI/HA-PEG/HA-M2pep nanoparticles to repolarize macrophages from M2 to M1 phenotype.

3.4 *In vivo* biodistribution of HA-PEI/HA-PEG-M2pep nanoparticles

To demonstrate that HA-PEI/HA-PEG-M2pep nanoparticles (NPs) can be delivered to the tumor area in vivo, we labeled the NPs with Cy5 dye. We evaluated the in vivo accumulation of HA-PEI-Cy5 NPs conjugated with M2 peptide or scramble peptide. In KPC mice bearing pancreatic tumors, we injected the mice intraperitoneally (i.p.) with HA-PEI-Cy5 NPs conjugated with, either M2 peptide o scramble peptide for 2 h. **Figure 4** shows that the NPs accumulated in pancreas and liver, however no significant difference was observed between the two formulations. Of note, no fluorescence was detected in spleen, kidney (**Figure 4**), brain or lungs (*not shown*). These data indicate that HA-PEI NPs can reach the pancreatic tumor area.

3.5 *In vivo* repolarization of M2 macrophages using HA-PEI/HA-PEG-M2pep nanoparticles encapsulating miR-125b

We then investigated the effect of of HA-PEI-miR-125b in repolarization of TAMs in KPC mice bearing pancreatic tumors, by assessing the phenotype of TAMs after intraperitoneal administration of miR-125b encapsulated in HA-PEI-M2pep. For this purpose, KPC mice bearing tumors (confirmed using ultrasound), were injected intraperitoneal (2x/week for 2 weeks) with HA-PEI- miR-125b NPs conjugated with M2 peptide or HA-PEI- scramble miRNA NPs conjugated with M2 peptide (**Figure 5A**). We assessed iNOS and Arg-1 expression levels of (as M1 and M2 markers, respectively) in the macrophage population isolated from the KPC pancreatic tumors by qPCR, and the Arg1expression levels by IHC.

Although the expression levels of F4/80+ macrophages in the tumor area was similar between the groups, we observed a 60% reduction in Arg1 levels ($p<0.01$) in tumors from KPC mice treated with HA-PEI-miR-125b NPs conjugated with M2 peptide, compared to the ones treated with HA-PEI- scramble miR (**Figure 5B-D**). Moreover, following i.p. administration of HA-PEI-miR-125b conjugated with M2 peptide for 2 weeks, there is a significant 4-fold increase in the iNOS/Arg1 ratio levels (M1/M2 markers) in the macrophage population isolated from the pancreatic tumors, compared to mice treated with HA-PEI-scramble miR conjugated with M2 peptide (**Figure 5E**). We additionally evaluated levels of cell surface based M1 and M2 macrophage polarization markers CD80, CD86 and CD163 as well as intracellular M2 macrophage polarization marker CD206 (**Figure 6A**). The tumor-associated macrophages

isolated from KPC mice treated with HA-PEI-miR-125b NPs conjugated with M2 peptide showed an increase in levels of M1 markers CD86 (~3.5-fold increase) and CD80 (~3-fold increase) compared to the mice treated with HA-PEI- scramble miR (**Figure 6B**). Additionally, the levels of both the M2 markers, CD206 and CD163 were significantly reduced in tumor-associated macrophages isolated from KPC mice treated with HA-PEI-miR-125b NPs conjugated with M2 peptide as compared to the mice treated with HA-PEI- scramble miR (**Figure 6B**). Collectively, these data suggest that HA-PEI-miR-125b conjugated with M2 peptide reprograms PDA-infiltrating TAMs from a M2-like phenotype toward an M1-like phenotype.

4. DISCUSSION

PDAC is a deadly disease in need for novel therapeutic strategies. A significant hurdle in PDAC treatment stems from the presence of a complex tumor microenvironment, consisting of a stromal shell rich in fibroblasts, endothelial cells, and the tumor core, consisting of tumor cells and infiltrated TAMs. In this proof-of-concept study, we evaluated a novel microRNA (miR)-based therapeutic paradigm for reprogramming TAMs from a pro-tumor M2 to anti-tumor M1 phenotype in KPC mice. We showed effective delivery, transfection, and TAM repolarization with miR-125b administered with HA-PEI/HA-PEG-M2 peptide nanoparticles in vitro and in vivo.

Macrophages are generally highly plastic cells, playing a crucial role in the innate and adaptive immune responses to pathogens and inflammatory stimuli. They can be activated and polarized to two distinct phenotypes: the classically activated (M1) and alternatively activated (M2) macrophages. During PDAC progression, TAMs switch to M2-like phenotype increasing fibroblastic morphology, upregulating mesenchymal markers, promoting proliferation and migration. Not surprisingly, M2-polarized TAMs infiltration in pancreatic cancer samples has a negative correlation with prognosis. Therefore, repolarization of TAMs from a predominant protumor (M2) to antitumor (M1) phenotype appears to be a promising strategy for the treatment of aggressive tumors, such as PDAC. In this study, we designed a novel strategy to specifically target M2 macrophages and reprogram them to M1-like phenotype using HA-PEI/HA-PEG-M2 peptide NPs nanoparticles for delivery of miR-125b.

Human PDAC arises from pancreatic intra-epithelial neoplasias, frequently driven by activating mutations in Kras [19], followed by loss or mutation of tumor suppressors, such as p53. To confirm our findings in vivo, we used the KPC genetically engineered mouse model. This model is based on the simultaneous Cre-dependent expression of $Kras^{G12D}$ and a dominant negative form of TP53 ($Trp53^{R127H}$) in the pancreas [15, 20]. Mice develop invasive PDA at ~10 weeks of age [18]. Metastases are observed in 80% of the KPC mice at the same sites observed in PDA patients (i.e., liver, lung, and peritoneum). Moreover, pancreatic tumors show many of the immunohistochemical markers associated with human PDA. Another crucial aspect of the KPC model is that the animals have a fully functional immune system, unlike in the immune-compromised xenograft models. In addition, KPC tumors recapitulate the dense stroma observed in human PDA. Based on the above, the KPC mouse model represents an excellent clinically relevant model to evaluate novel therapeutic strategies, including TAMs repolarization [21].

Delivery of nucleic acid poses significant challenges, such as poor *in vivo* half-life, cell penetration incapability, and instability within the cell endosomes. Among various delivery systems, non-viral vectors have been embraced for gene delivery applications primarily due to their safety, versatility and ease of production, application, and scale-up ability [9]. Actively targeting RNA nano-carriers engineered from biocompatible polymers have unique properties that can be tailored by controlling polymer composition, size, shape, and surface characteristics [22]. Furthermore, following systemic administration, nanoparticles (NPs) may accumulate in tumor tissues through the enhanced permeability and retention effect (EPR) [23]. Once NPs accumulate in the tumor tissue, the regulation of TAM functions may be restricted within tumor microenvironment, hence reducing systemic toxicity.

We have anchored the cationic polymer, poly(ethylene imine) (PEI) on to the HA backbone, which enables self-assembly of the conjugate in the presence of negatively charged nucleic acids based on electrostatic interactions. Importantly, chemical attachment of PEI on the HA backbone mitigates the cytotoxicity that is often associated with free PEI. Apart from using polymers for targeted delivery, several papers have reported surface functionalization of NPs using targeting ligands such as folic acid and epidermal growth factor peptide to achieve tumor specific delivery [24]. Cieslewicz et al have previously shown the use of M2 peptide to specifically target TAMs (M2 macrophages) [14]. We have successfully conjugated this M2

peptide HA-PEG polymer and evaluated the HA-PEI/HA-PEG-M2 peptide nanoparticle system to target TAMs. This dual targeting strategy with HA and M2 peptide led to enhanced uptake by M2 macrophages as compared to the untargeted nanoformulations which serves as an added advantage over other HA-PEI based nanoformulations [11-12, 25]. TAMS specific We had previously showed that intraperitoneal administration of HA-PEI nanoparticles would bring about effective reprogramming of tumor associated macrophages to anti-tumoral phenotype in turn reducing the tumor burden in an ovarian cancer mice model [13]. Based on the above evidence we used the similar dosing strategy of intraperitoneal administration of M2 peptide targeted HA-PEI nanoparticles in the pancreatic tumors. Additionally, using intraperitoneal administration we were able to increase the dosing volume as well as reduce potential toxicity. Thus, following i.p administration, we observe that HA-PEI/HA-PEG-M2 peptide nanoparticle encapsulating miR-125b reach the tumor site and reprograms PDA-infiltrating TAMs toward an M1-like phenotype, as suggested by the reduction in Arg1, CD206 and CD163 levels as well as increase in CD80 and CD86 levels. Similarly, it has previously been documented that selective macrophage transfection with miR-125b using HA-PEI nanoparticles can also effectively increase in the M1 to M2 macrophage ratio and improved anti-tumor efficacy in a non-small cell lung cancer and ovarian cancer mouse models [12, 13], highlighting that this system is not tumor restrictive.

5. CONCLUSION

In summary, we have developed a novel miR-based therapeutic paradigm for reprogramming TAMs from a pro-tumor M2 to anti-tumor M1 phenotype. We showed effective delivery, transfection, and TAM repolarization with miR-125b administered with HA-PEI/HA-PEG-M2 peptide NPs in cells and in KPC mice, strongly suggesting that intraperitoneal administration of HA-PEI/HA-PEG-M2 peptide miR-125b nano-formulations is an effective strategy for repolarization of TAMs. Further studies are warranted to evaluate the efficacy of this novel treatment strategy alone and in combination with current chemotherapeutic drugs as a potential anticancer therapy in PDAC.

6. ACKNOWLEDGEMENTS

Financial support for this work was provided by the United States National Cancer Institute of the National Institute of Health through grants R21-CA213114-01 and the Northeastern University-Dana Farber Cancer Center Joint Program on Cancer Drug Development.

7. REFERENCES

1. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer statistics, 2020.* CA Cancer J Clin, 2020. **70**(1): p. 7-30.
2. Hao, N.B., Lü, M. H., Fan, Y. H., Cao, Y. L., Zhang, Z. R., Yang, S. M., *Macrophages in tumor microenvironments and the progression of tumors.* Clinical and Developmental Immunology, 2012. **2012**.
3. Genard, G., S. Lucas, and C. Michiels, *Reprogramming of Tumor-Associated Macrophages with Anticancer Therapies: Radiotherapy versus Chemo- and Immunotherapies.* Front Immunol, 2017. **8**: p. 828.
4. Squadrito, M.L., Etzrodt, M., De Palma, M., Pittet, M. J., *MicroRNA-mediated control of macrophages and its implications for cancer.* Trends in Immunology, 2013. **34**(7): p. 350-359.
5. Liu, C.Y., et al., *M2-polarized tumor-associated macrophages promoted epithelial-mesenchymal transition in pancreatic cancer cells, partially through TLR4/IL-10 signaling pathway.* Laboratory Investigation, 2013. **93**(7): p. 844-854.
6. Mielgo, A. and M.C. Schmid, *Impact of tumour associated macrophages in pancreatic cancer.* Bmb Reports, 2013. **46**(3): p. 131-138.
7. Chaudhuri, A.A., et al., *MicroRNA-125b potentiates macrophage activation.* J Immunol, 2011. **187**(10): p. 5062-8.
8. Glover, D.J., H.J. Lipps, and D.A. Jans, *Towards safe, non-viral therapeutic gene expression in humans.* Nat Rev Genet, 2005. **6**(4): p. 299-310.
9. Misra, S., Hascall, V.C., Markwald, R.R. and Ghatak, S., . Interactions between hyaluronan and its receptors (CD44, RHAMM) regulate the activities of inflammation and cancer. Frontiers in immunology, 2015. 6, p.201.
10. Ganesh, S., Iyer, A. K., Weiler, J., Morrissey, D. V., Amiji, M. M., *Hyaluronic acid based self-assembling nanosystems for CD44 target mediated siRNA delivery to solid tumors.* Biomaterials, 2013. **34**(13): p. 3489-3502.
11. Ganesh, S., Iyer, A. K., Weiler, J., Morrissey, D. V., Amiji, M. M., *Combination of siRNA-directed gene silencing with cisplatin reverses drug resistance in human non-small cell lung cancer.* Molecular Therapy - Nucleic Acids, 2013. **2**(1).
12. Parayath, N. N., Parikh, A., & Amiji, M. M. Repolarization of tumor-associated macrophages in a genetically engineered nonsmall cell lung cancer model by intraperitoneal administration of hyaluronic acid-based nanoparticles encapsulating microRNA-125b. *Nano letters*, 2018. ***18***(6), 3571-3579.

13. Parayath, N. N., Gandham, S. K., Leslie, F., & Amiji, M. M. Improved anti-tumor efficacy of paclitaxel in combination with MicroRNA-125b-based tumor-associated macrophage repolarization in epithelial ovarian cancer. *Cancer letters*, 2019. ***461***, 1-9.
**14. Cieslewicz, M., et al., *Targeted delivery of proapoptotic peptides to tumor-associated macrophages improves survival.* Proceedings of the National Academy of Sciences, 2013. *This is the original study that describes M2 peptide. We have used the M2 peptide sequence described in this study for formulating our targeted HA-PEI-/HA-PEG nanoparticles.*
15. Hingorani, S.R., et al., *Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice.* Cancer Cell, 2005. **7**(5): p. 469-83.
16. Gopinathan, A., et al., *GEMMs as preclinical models for testing pancreatic cancer therapies.* Dis Model Mech, 2015. **8**(10): p. 1185-200.
17. Mackenzie, G.G., et al., *Phospho-sulindac (OXT-328) combined with difluoromethylornithine prevents colon cancer in mice.* Cancer Prev Res (Phila), 2011. **4**(7): p. 1052-60.
18. Mallangada, N.A., et al., *A novel tricarbonylmethane agent (CMC2.24) reduces human pancreatic tumor growth in mice by targeting Ras.* Mol Carcinog, 2018. **57**(9): p. 1130-1143.
19. Almoguera, C., et al., *Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes.* Cell, 1988. **53**(4): p. 549-54.
20. Olive, K.P. and K. Politi, *Translational therapeutics in genetically engineered mouse models of cancer.* Cold Spring Harb Protoc, 2014. **2014**(2).
21. Lee, J.W., et al., *Genetically Engineered Mouse Models of Pancreatic Cancer: The KPC Model (LSL-Kras(G12D/+) ;LSL-Trp53(R172H/+) ;Pdx-1-Cre), Its Variants, and Their Application in Immuno-oncology Drug Discovery.* Curr Protoc Pharmacol, 2016. **73**: p. 14 39 1-14 39 20.
22. Abeylath, S.C., et al., *Combinatorial-designed multifunctional polymeric nanosystems for tumor-targeted therapeutic delivery.* Acc Chem Res, 2011. **44**(10): p. 1009-17.
23. Nguyen, C.T., et al., *Redox-sensitive nanoparticles from amphiphilic cholesterol-based block copolymers for enhanced tumor intracellular release of doxorubicin.* Nanomedicine, 2015.
24. Talekar, M., Kendall, J., Denny, W., Garg, S., *Targeting of nanoparticles in cancer: Drug delivery and diagnostics.* Anti-Cancer Drugs, 2011. **22**(10): p. 949-962.
25. Sahiner, N., Sagbas, S., Sahiner, M. and Ayyala, R.S. *Polyethyleneimine modified poly (Hyaluronic acid) particles with controllable antimicrobial and anticancer effects.* Carbohydrate polymers, 2017. 159, pp.29-38.

FIGURE LEGENDS

Figure 1: (A) Synthesis and cancerization of hyaluronic acid-poly(ethylene imine) (HA-PEI), HA-poly(ethylene glycol) (HA-PEG) and HA-PEI/HA/PEG-Peptide conjugates. HA-PEI and HA-PEG-maleamide derivatives were synthesized using a simple and versatile EDC/NHS conjugation chemistry. M2 peptide is further conjugated to HA-PEG-maleimide. Both the HA

polymers are used in 1:1 ratio for encapsulation of miRNA-125b. (B) The ^{1}H-NMR spectra of the peptide show the characteristic aromatic ring peaks between 6.5 -7.5 ppm. The 1H-NMR spectra of the HA-PEG-Peptide also shows the characteristic aromatic ring peaks between 6.5 - 7.5 ppm. (c) Schematic representation of HA-PEI/PEG M2 Peptide miRNA nanoparticle formation (d) Hydrodynamic diameters, polydispersity index (PDI) and ζ-potentials of HA-PEI/ HA-PEG-Scram peptide or HA-PEI/HA-PEG-M2 peptide nanoparticle formulations. n = 4 identically prepared samples, data are expressed as mean ± SD.

Figure 2: (A) J774 murine macrophages cells were treated with IL-4 (100 ng/ml) for 6 h, followed by treatment with Cy5 labeled HA-PEI/HA-PEG-Scram peptide or HA-PEI/HA-PEG-M2 peptide nanoparticles for 2 h, 4 h, 6 h and 12 h. Representative images of cells stained with DAPI for confocal analysis. Scale bar indicates 50 μm. (B) J774 murine macrophages cells were treated with IL-4 (100 ng/ml) for 6 h, followed by treatment with Cy5 labeled HA-PEI/HA-PEG-Scram peptide or HA-PEI/HA-PEG-M2 peptide nanoparticles for 4 h, 6 h, 12 h and 20 h. Representative histograms of cells after FACS analysis are shown.

Figure 3: *In vitro* polarization of J774 macrophages using miR125b encapsulated HA-PEI/ HA-PEG-peptide. (A) Schematic method for *in vitro* macrophage repolarization study. Expression of (B) Arg-1, (C) iNOS and (D) iNOS/Arg-1 ratio in IL-4 stimulated macrophages treated with HA-PEI/HA-PEG-Scram peptide or HA-PEI/HA-PEG-M2. qPCR was used for quantification of gene expression level with β-actin as the internal control, *$p<0.05$, n =4.

Figure 4: Biodistribution of HA-PEI/PEG Nanoparticles in KPC mice. (A) Schematic method for biodistribution of HA-PEI/PEG Nanoparticles in KPC mice. Presence of Cy5 labeled HA-PEI NPs formulations (M2 peptide- or scramble peptide-conjugated) in KPC pancreas. (B) Representative images of the pancreas, liver, spleen and kidney tissues were isolated from 12-week old KPC mice treated with i.p. administration HA-PEI-Cy5 (M2 peptide- or scramble peptide-conjugated) for 2 h. (C) The fluorescence of Cy5 was determined through imaging by the IVIS system.

Figure 5: *In vivo* polarization of TAMs using miR125b encapsulated HA-PEI/HA-PEG-peptide in KPC mice. (A) Schematic method for *in vivo* macrophage repolarization study in KPC mice. (B) F4/80 and (C) Arg1 immunostaining were performed on KPC tumor sections and photographs were taken at x20 (for F4/80) and x10 (for Arg-1) magnification. (D) Representative images are shown. The consecutive section was stained with isotype IgG as negative staining control and it is shown in the upper right corner. Results were expressed as percent of F4/80+ or Arg1+ cells ± SD per x20 or x10 field, respectively. (e) The ratio of iNOS/Arg1 in KPC tumor macrophages treated with HA-PEI nano-formulations. qPCR was used for quantification of the gene expression level with β-actin as the internal control. *Significant compared to control group; $P < 0.01$.

Figure 6: Characterization of TAMs using miR125b encapsulated HA-PEI/HA-PEG-peptide in KPC mice. (A) Schematic method for characterization of TAMs in KPC mice. (B) Expression levels of CD80, CD86, CD163 and CD206 in TAMs isolated from pancreatic tumor tissue of KPC mice treated with HA-PEI/PEG M2 peptide miR125b nano-formulations or scrambled miR nanoparticles. qPCR was used for quantification of the gene expression level with β-actin as the internal control. *Significant compared to control group; $P < 0.01$.