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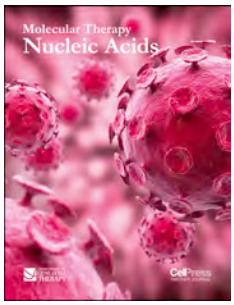

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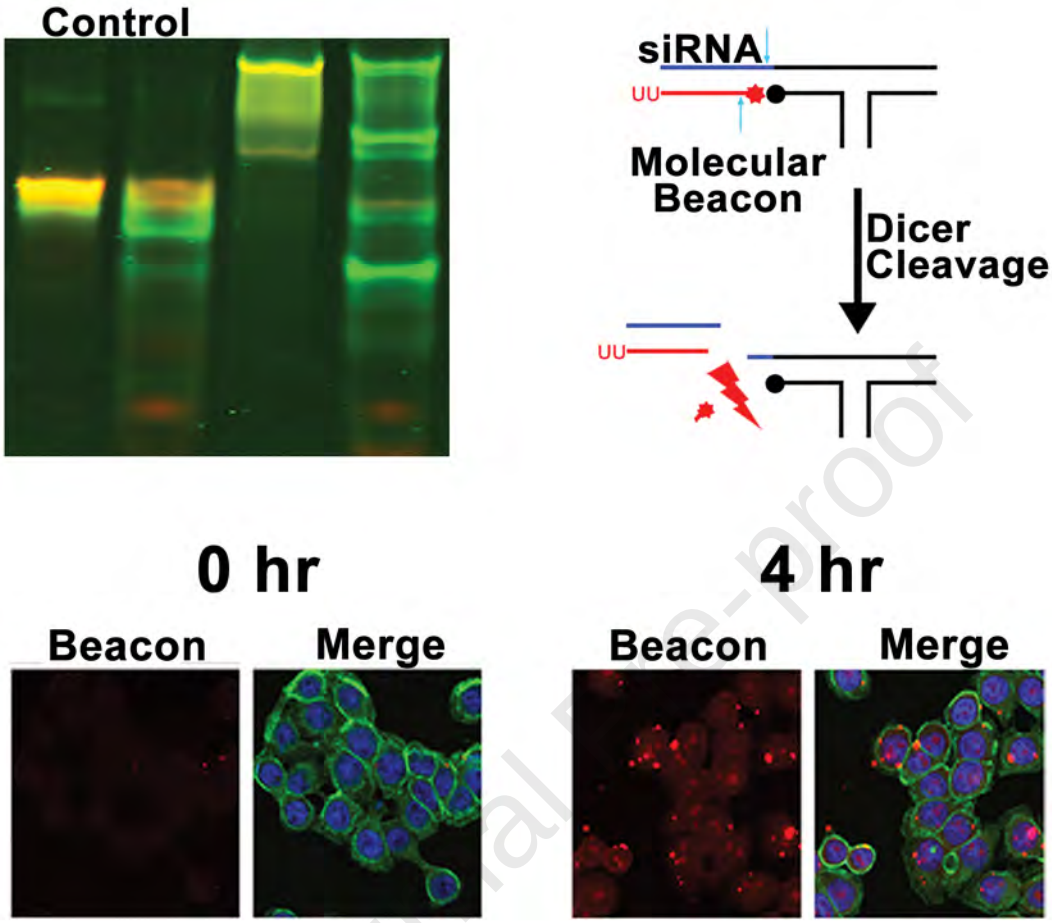

Rational Design for Controlled Release of Dicer-substrate SiRNA Harbored in phi29 pRNA-based Nanoparticles

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Short Title: Controlled Release of siRNA from RNA Nanoparticles

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Abstract

SiRNA for silencing genes and treating disease has been a dream since ranking as a top Breakthrough of the Year in 2002 by *Science*. With the recent FDA approval of four siRNA-based drugs, the potential of RNA therapeutics to become the third milestone in pharmaceutical drug development has become a reality. However, the field of RNAi therapeutics still faces challenges such as specificity in targeting, intracellular processing, and endosome trapping after targeted delivery. Dicer-substrate siRNAs included onto RNA nanoparticles may be able to overcome these challenges. Here we show that pRNA-based nanoparticles can be designed to efficiently harbor the Dicer-substrate siRNAs for *in vitro* and *in vivo* to the cytosol of tumor cells and release the siRNA. The structure optimization and chemical modification for controlled release of Dicer-substrate siRNAs in tumor cells were also evaluated through molecular beacon analysis. Studies on the length requirement of the overhanging siRNA revealed that at least 23 nucleotides at the dweller's arm were needed for dicer processing. The above sequence parameters and structure optimization were confirmed in recent studies demonstrating the release of functional Survivin siRNA from the pRNA-based nanoparticles for cancer inhibition in non-small-cell lung, breast, and prostate cancer animal models.

Introduction

RNA interference (RNAi) is a post-transcriptional gene regulation pathway used by different classes of small RNAs.[1] Among them, small interfering RNAs (siRNA)[2] have attracted attention for their important potentials in drug discovery and development. siRNA target and bind mRNA to produce gene silencing effects, but their mechanisms are distinct.[3] SiRNA, for its ability to silence genes and potential in treating diseases, has been a popular dream since *Science* ranked this technology as a top ten Breakthrough of the Year in 2002.[4] siRNA was first discovered in 1998 in *C. elegans* and in mammals in 2001.[5, 6] Since many researchers have worked to bring siRNA to the clinic until the recent FDA approval of Alnylam's Onpattro, the first siRNA drug.[7, 8] Shortly thereafter the FDA has approved Givlaari and Oxlumo that were also developed and produced by Alnyam.[9-11] Such FDA approvals have moved towards RNA becoming the third milestone in pharmaceutics, following chemical and protein based therapies.[12] However, the full potential of siRNA into mainstream therapeutics has not been realized. Further strategies are needed to avoid endosomal trapping.

siRNA is synthetically created, short RNA with matching sequence to its corresponding mRNA. When internalized into cells, siRNA is incorporated into the RNA-induced silencing complex (RISC), like microRNA.[13] Classical siRNAs are short and typically only 19 bp in length, although longer ones exist; alternatively, longer Dicer-substrate siRNAs range from 20-27 bp and require Dicer processing.[14] Both classical and Dicer-substrate siRNAs interact with and activate RISC. The sense strand of siRNA is degraded,[15] while the anti-sense strand associated with RISC recognizes its target mRNA for cleavage by AGO2. However, the longer Dicer-substrate siRNAs require Dicer processing before loading into RISC. It is reported that

Dicer-substrate RNA duplexes have increased gene silencing activity compared to classic 19-bp siRNAs.[16]

For RNA to be considered for clinical applications it must be stable *in vivo*, thus demanding stability against nucleases. In order to increase nuclease stability and reduce immunogenicity, nucleotide sugar modifications can be used (most commonly 2'-Fluoro (2'-F), 2'-OMethyl, or 2'-O-methoxyethyl (2'-MOE)); however, Dicer-substrate siRNAs do not tolerate sugar modifications at each position, since these modifications can interfere with Dicer recognition. Therefore, careful consideration must be taken to increase siRNA stability. Researchers have created nuclease stable siRNA while remaining sensitive to Dicer by only modifying key nucleotide sites against nucleases and reducing off-target effects.[17-21] 2'F, 2'-OMethyl modifications on the sense strand and at the termini of anti-sense strands retained Dicer processing. Additionally, 2'-MOE modifications were very site-specific due to the bulky size of the modification. However, heavy modification of the anti-sense strand resulted in Dicer inactivity. Understanding and exploiting Dicer processing is of significance in the design and optimization of siRNA for efficient gene knockdown.

The Dicer enzyme belongs to the RNase III family, which can cleave long double-strand RNAs into small RNAs.[22] It is reported that the structure of the human Dicer is L shaped and composed of a head, body and base.[13, 22, 23] The head is the PAZ (piwi/ argonaute/ zwille) domain, which is also the RNA binding domain. PAZ domain has a high affinity for a 3' protruding 2 nt overhang on RNAs. Two RNase III domains are located in the "body" and form the processing center, where each RNAase III cleaves one of the long double-strand RNA. And the base is an N-terminal DExD/H-box helicase domain with a clamp-shaped structure. Dicer can cleave long ds-RNA into 21-22 bp ds-RNA. Dicer itself is like a molecular ruler since the

distance between the binding PAZ domain and cutting RNase III domain is about the length of 21-22 bp siRNA.[24, 25] Many factors affecting Dicer processing have been investigated, including mutations to the PAZ domain responsible for RNA recognition, and in the design of siRNA in relation to the 5'/3' structuring for Dicer binding and inclusion of chemical modifications (discussed above).[26-29] However, some conclusions are inconsistent or contradictory and seem to be specific to each siRNA sequence.

To achieve *in vivo* gene knockdown, another key is efficient delivery of RNAi into cancer cells. Although many nanocarriers have been developed, our studies[12, 30, 31] have shown that RNA nanotechnology provides one of the best strategies to deliver RNAi into cancer cells.[32-34] By definition, RNA nanotechnology is the bottom-up construction of nanostructures composed primarily of RNA, including the core scaffolding and any functional group attached to the nanoparticle.[34] Since its conception, more and more complex RNA nanoparticles have been constructed with high thermodynamic stability and proven to function well in *in vivo* applications.[32, 35-38] Our RNA nanoparticles, using the phi29 DNA packaging motor pRNA as a core motif, are homogeneous in size, structure, and stoichiometry; are thermodynamically and chemically (after 2'-F modifications) stable;[36, 39-42] retain authentic folding and independent functionalities of all incorporated modules (RNA aptamer, siRNA, miRNA or ribozyme);[36, 43] are non-toxic,[42, 44] highly soluble, and display favorable biodistribution and PK/PD profiles.[36, 39, 42, 44-46] Furthermore, the application of RNA nanoparticles to exosomes, 30-150 nm membranous vesicles of endocytic origin as a way of intercellular communications,[47, 48] allows for both the loading of RNA nanoparticle-siRNA cargos and displaying tumor targeting ligands.[30] Exosomes have an endomembrane-like membrane property (structure, lipid, peptides, protein, etc.) and have been shown to carry genetic materials, especially RNA, as a form of intercellular

communication.[47-54] Exosomes have been considered for their therapeutic applications due to their favorable size and are well-tolerated *in vivo.*[51] Therapeutic payloads, such as siRNA, can remain fully functional after delivery to cells by exosomes.[47, 48, 52, 55] These exosomes have demonstrated their ability to fuse to the cell membrane of the targeted cells resulting in the dumping of their cargo directly into the cytosol of cells.[56] As such we utilize these exosomes for their ability to avoid endosome trapping and therefore increase the efficiency of delivered siRNAs.[56]

RNA nanotechnology has gained significant interest in the development of novel nano-structures.[57-64] Several researchers have used these RNA nanoparticles for the inclusion of RNAi components, including siRNAs, and examined their specific gene silencing abilities.[65-70] Similar to our approaches of applying RNA nanoparticles to exosomes, complex delivery vehicles have been created by functionalizing RNA nanoparticles with polymer nanoparticles[71] and exosomes[72] for improved siRNA delivery. Thus RNA nanoparticles are poised to overcome the previous roadblocks in siRNA therapies. However, little is known on how the delivered siRNA by RNA nanoparticles and exosomes interacts with the Dicer complex to achieve efficient knockdown of target genes.

In the present study we examine if Dicer-substrate siRNA can be incorporated onto pRNA based RNA nanoparticles and their required design parameters to retain Dicer processing. We integrate and test factors affecting Dicer processing, which directed us to design siRNA loaded RNA nanoparticles that can function as a Dicer substrate to generate a robust gene regulation tool. Dicer-substrate siRNA is used in these studies due to the ease of inclusion into RNA nanoparticles by simple helical extension, increased efficacy in gene knockdown over short siRNA, and reduced immune responses over short siRNA.[14, 16] Unlike shorter siRNA, Dicer-

substrate siRNA has not translated to the clinic due to delivery issues and requirements of Dicer processing, here we aim to overcome these issues. Besides inclusion of 3'- two nucleotides overhangs and a 5'-phosphate group, we also investigated whether chemical modifications affect Dicer processing. Based on the screened factors, Luciferase 2 (*LUC2*) siRNA was designed to be incorporated into RNA nanoparticles. To demonstrate the Dicer processing, a molecular beacon was designed and constructed to monitor the activity. Our findings suggest that an integration of length, nucleotide components, structure, and RNA nanotechnology into the designs of RNAi achieves efficient silencing of targeted genes in an *in vivo* cancer therapy setting. As such our findings provide design parameters for siRNA incorporation into RNA nanoparticles for efficient gene silencing.

Results

RNA nanoparticles harboring siRNA were processed by Dicer into functional siRNA resulting in cleavage of target gene

RNA nanoparticles based on the phi29 pRNA were constructed to include Lamin25 siRNA extended on the pRNA 5'/3' terminal end (pRNA-Lamin25). Lamin A/C was chosen as a model gene to demonstrate the specific cleavage of mRNA by a 5' RACE (rapid amplification of cDNA ends) technique to prove that the gene silencing activity of pRNA nanoparticle is mediated by RNA interference.[73] pRNA-Lamin25 was designed similar to that described by Zhang *et al*,[74] with 25 bp siRNA linked to a pRNA vector by a double uracil (-UU-) linker (**Fig. 1A**). RNA was prepared by *in vitro* transcription by T7 RNA polymerase and transfected into KB cells. The gene silencing efficiency of pRNA-Lamin25 was first demonstrated by quantitative qRT-PCR (**Fig. S1**). A RNA ligation step and two rounds of PCR was used to reveal the sequence of

mRNA after cleavage. A DNA fragment representing the cleaved mRNA was detected in the pRNA-Lamin25 sample with the size between 200 bp and 300 bp in gel electrophoresis (**Fig. 1B**). The mRNA cleavage was expected to be 243 bp in length indicating successfully cleaved Lamin A/C. Sequencing results of the excised DNA fragment confirmed that Lamin A/C mRNA was cleaved at a position ten nucleotide from the 5' end of the antisense of Lamin25 siRNA sequence as predicted. No fragment was detected in the control samples treated with pRNAi-scramble (**Fig. 1B-lane 2**). These data demonstrate that gene silencing mediated by the pRNA-siRNA is induced by RNAi pathway as in standard siRNA duplex. The incorporation of siRNA into RISC and cleaving the Lamin A/C mRNA proves RNA nanoparticles are able to deliver siRNA sequences into cancer cells and specifically silence its respective gene.

Development of RNA nanoparticle molecular beacons for observation of in vitro processing and release of siRNA

Based on the above results, it was determined that siRNA sequences can release from RNA nanoparticles within the cells. However, confirmation of Dicer processing of siRNA from RNA nanoparticles has not been observed, nor is it understood the release mechanism of siRNA from the RNA nanoparticles. pRNA-3WJ RNA nanoparticles were constructed incorporating 24 bp, 3'-UU overhang luciferase siRNA oligo (*LUC2*-siRNA). The pRNA-3WJ motif was used in these studies due to ease of construction and the multivalent nature of the pRNA-3WJ allows for inclusion of siRNA on one helical branch while targeting ligands, such as RNA aptamers, can be added to other branches to fulfill specific and favorable cancer targeting.[36, 44, 45, 75] This design takes advantage of the high chemical and thermodynamic stability as well as multivalency of the 3WJ scaffold for therapeutics loading and delivery.[38, 76] To demonstrate that the siRNA

178 incorporated to 3WJ RNA nanoparticles could be processed by Dicer for higher gene regulation
179 efficacy, a molecular beacon was designed accordingly by introducing a fluorophore/quencher
180 pair into the nanoparticles (**Fig. 2A**). A Cy5 fluorophore was conjugated to 5'-end of antisense
181 strand while a BBQ650 fluorescence quencher was attached to 3'-end of 3WJ-c strand adjacent
182 to the Cy5 label. Such design quenches the Cy5-siRNA signal until the siRNA is processed by
183 Dicer, thus creating a short, Cy5-labeled sequence on the 3WJ nanoparticle that is quickly
184 dissociated due to lack of strong base pairing resulting in recovery in fluorescent signal.

185 Stepwise assembly of the 3WJ/*LUC2*-siRNA was confirmed by 15% polyacrylamide gel in
186 native folding conditions, and incorporation of BBQ650 resulted in high quenching of the Cy5
187 signal (**Fig. 2B**). Serum stability and Temperature Gradient Gel Electrophoresis (TGGE) studies
188 proved resulting nanoparticles that have high chemical and thermodynamic stability, respectively
189 (**Fig. 2C** and **2D**), which guarantees the low background noise signal due to lack of *in vivo*
190 degradation of the nanoparticles.

191 This 3WJ-*LUC2*-siRNA beacon design was used to monitor Dicer processing by intracellular
192 imaging. When the nanoparticle is intact, Cy5 signal is quenched by the BBQ650 due its close
193 proximity and low fluorescence could be detected ("OFF" status). The Cy5 fluorescence is
194 restored when the nanoparticles underwent processing by Dicer and the short fragment with Cy5
195 fell off of the nanoparticle creating distance with the BBQ650 ("On" status) (see **Fig. 2A**). By
196 comparing the fluorescence intensities between 3WJ/*LUC2*-siRNA-Cy5 and 3WJ/*LUC2*-siRNA-
197 Cy5/BBQ650 (molecular beacon), we have a better understanding about the processing of the
198 siRNA in the cell cytosol and its processing efficiency.

199

200 ***Direct observation of in vitro siRNA processing from RNA nanoparticles***

Exosomes are lipid vesicles released from cells typically for intercellular communications.[77] We previously modified the isolated exosomes from HEK293 cells with RNA nanoparticles, and demonstrated that RNA displaying exosomes are able to deliver loaded cargos directly to the cytosol of cells.[30, 56, 78] HEK293 exosomes were previously shown to have an average size of 96 nm via Nanoparticle Tracking Analysis, a negative zeta potential of -4.6 mV via Dynamic Light Scattering, and expression of TSG101, a typical exosome specific marker.[79] With concerns of cytosol trapping, thus preventing siRNA from releasing into the cytosol, we loaded 3WJ/*LUC2*-siRNA beacon nanoparticles into exosomes to be delivered to HT29 cells with continuous luciferase expression (HT29-luc). Additionally, the exosomes were decorated with previously developed RNA nanoparticles harboring folate to provide specific targeting and binding to the folate receptor expressing HT29 cells.[30] Confocal fluorescent microscopy and flow cytometry (FACS) demonstrated high binding efficiency of folate targeting exosomes (FA/EV/*LUC2*-siRNA/Cy5) to the HT29-luc cells (**Figure S2A**) and internalization of the 3WJ/*LUC2*-siRNA/Cy5 loaded into the exosomes over controls (**Fig. S2B**). A clear shift in peak fluorescence of the HT29 cells was seen in both FACS and confocal studies of the FA/EV/*LUC2*-siRNA/Cy5 over the 3WJ/EV/*LUC2*-siRNA/cy5 with nearly triple the fluorescence intensity seen in the FACS results.

Utilizing the exosome delivery vehicle allowed the 3WJ/*LUC2*-siRNA beacon nanoparticles to be efficiently distributed to the cytosol of the cancer cells for a time-course study of siRNA processing by Dicer. As a positive control to demonstrate Dicer processing rather than random RNase degradation, a truncated 19 bp *LUC2*-siRNA was placed onto the 3WJ (S1: 3WJ/*LUC2*-siRNA-tru) (**Fig. 3A**). 3WJ/*LUC2*-siRNA/Cy5 (S3) nanoparticles were also tested as a total Cy5 signal control. 3WJ/*LUC2*-siRNA beacon (S2) as well as control nanoparticles were delivered by

the folate displaying exosomes. The processing of siRNA from 3WJ scaffold was reflected by Cy5 fluorescence gradual increase in signal over time, as shown in confocal microscopy studies (**Fig. 3B-3D**). As such the data directly demonstrated that Dicer is able to bind and cleave siRNA incorporated into RNA nanoparticles. This proved that the RNA nanoparticle motif does not interfere with Dicer siRNA processing due to steric hindrance or processing issues as a result of the nanoparticles' branched nature. It is noted that fluorescence in the 3WJ/*LUC2*-siRNA/Cy5 positive control, fluorescence intensity increases over time due to the longer incubation time of the RNA nanoparticle/exosome complex with cells. This allows for increased levels of 3WJ/*LUC2*-siRNA/Cy5 to be released in the cells resulting in higher fluorescence levels over time. However, such control serves as a siRNA delivery vehicle control to ensure that siRNA is being delivered within cells.

In vitro identification of the specific cleavage site of siRNA by Dicer as RNA nanoparticle design principle

In order to better understand the Dicer processing of siRNA from our RNA nanoparticles and provide design principles for siRNA incorporation onto RNA nanoparticles, we examined Dicer processing to various siRNAs *in vitro*. Rational design of siRNA should be illustrated when considering incorporation to RNA nanoparticles to ensure the intracellular Dicer processing of siRNA. Therefore, different factors affecting Dicer processing including siRNA length, 5'-phosphate, 3'-two nucleotides overhang, and 2'-F modifications were tested based on the Luciferase2-siRNA used above. The base pairing length is a key factor as Dicer processes siRNA according to the loop-counting rule.[80] DsRNA substrate is anchored to the PAZ domain and the specified distance between the anchored helical end of the dsRNA and the RNase active

site in the RNaseIII domains serves as a molecular ruler.[2, 27, 81] To test the cleavage based on the loop-counting rule, different lengths (19 bp, 21 bp, 22 bp, 23 bp and 24 bp) of *LUC2*-siRNAs were designed and constructed (**Table S1**) to be tested for Dicer processing using Human Recombinant Dicer Enzyme. After incubation with the Dicer enzyme, 3WJ-siRNA conjugates were assayed by polyacrylamide gel looking for a cleaved product. The results demonstrated that siRNA longer than 21 bp showed obvious processing, and 24 bp *LUC2*-siRNA could be processed with the highest efficiency (**Fig. 4A**). Shortening either strand of the double-stranded *LUC2*-siRNAs resulted in inhibition of Dicer processing (**Fig. 4B**), which is due to the dimer structure of RNaseIII domain. In addition, siRNA constructs regarding 5'-phosphate, 3'-UU overhang and 2'-F modification were tested for Dicer processing (**Fig. 4C**). 2'-F modifications were selected as they are well studied in our pRNA-3WJ system and have shown not to affect the 3WJ structuring while increasing thermodynamic and enzymatic stabilities.[38, 76] Quantification of Dicer processed bands demonstrated that the absence of 3'-UU reduces Dicer processing efficiency to only 62% (**Fig. 4C lane 5**), which may be due to less favorable Dicer enzyme binding. When including enzyme stabilizing chemical modifications, 2'-F modifications to the pyrimidines of the siRNA sense strand also decreased the processing to 45% (**Fig. 4C lane 3**); however, the presence of a 5'-phosphate to the sense strand did not affect Dicer processing much in our testing (**Fig. 4C lane 2&4**). While the decreased Dicer processing in adding 2'-F modifications is disappointing, these modifications are typically required for *in vivo* applications for the increased thermodynamic and enzymatic stabilities of the RNA nanoparticles. However, the incorporation of the 3'-UU overhang of the anti-sense strand significantly improves Dicer processing.

From our testing we were able to further demonstrate loading of siRNA onto RNA nanoparticles still allows for Dicer recognition and processing of the siRNA (**Fig. 4D**). The use of the molecular beacon demonstrated that 24 bp siRNA is cleaved from the nanoparticle resulting in Cy5 signal. Dicer processing of various siRNA designs indicated that when incorporating siRNA onto RNA nanoparticles, the length of the siRNA must remain at a minimum length of 22 bp.

Functional siRNA releasing from the RNA nanoparticles has been confirmed in multiple animal trials on lung, breast, and prostate cancer models.

Furthermore, our group has proven the *in vivo* delivery of siRNA via RNA nanoparticles harboring Survivin siRNA loaded into exosomes (**Fig. 5**).[30, 82] Microscopy studies demonstrated that the exosomes allow for membrane fusion and cytosol dumping of therapeutic cargos,[56] while resulting in ultra-high inhibition of tumor growth in *in vivo* tumor models.[30] While it is thought that endosome entrapment can allow for the slow release of stable siRNA into the cells,[83] our exosomes avoid endosome entrapment and create direct siRNA delivery to the cytosol of targeted cells for swift treatment.[56]

The releasing of functional siRNA from our RNA nanoparticles was documented by animal trials in multiple publications showing the inhibition of tumors in non-small-cell lung (NSCLC),[82] triple negative breast (TNBC),[30] and prostate cancer[30] models when Survivin siRNA was fused onto RNA nanoparticles and delivered to tumors (**Fig. 5**). Prostate specific membrane antigen (PSMA) RNA aptamers allowed for specific targeting and delivery of the siRNA to PSMA+ prostate xenograft tumors in nude mice using LNCaP-LN3 cells.[30] Additionally, epidermal growth factor (EGFR) RNA aptamers were displayed on the surface of exosomes for

delivery to orthotopically developed TNBC tumors using MDA-MB-468 cells[30] and separately NSCLC xenograft tumors using H596 cells.[82] Exosomes were delivered intravenously via tail-vein injection. **Figure 5** demonstrated that, across three tumor models, exosomes modified with RNA nanoparticles are able to deliver Survivin siRNA that is then processed in the tumors resulting in strong tumor inhibition. Full data on these studies can be found in Pi, *et al. Nat Nanotechnol*, 2018; 13(1):82-89 and Li *et al. Nucleic Acid Ther* 2021; ahead of print.[30, 82]

Discussion

Since its discovery in 1999,[5] siRNAs and other RNAi sequences have shown great promise in treating diseases and cancers by silencing specific responsible genes. Many large pharmaceutical companies and research groups have invested billions of dollars towards developing siRNA and other RNAi technologies for the treatment of cancers.[84-88] However, difficulties in producing a safe and efficient delivery system for the siRNA resulted in diminishing interest. Yet the promise of siRNA for the treatment of cancers and viral-infections still remains.[89] There is a desperate need for more efficient delivery vehicles that not only specifically target disease sites with high affinity but also deliver the RNAi payloads for Dicer processing.

In the presented study, the pRNA based RNA nanoparticles resulted in the specific knockdown and gene silencing via siRNA delivery. Our cell microscopy studies involving siRNA beacons conjugated to RNA nanoparticles demonstrate a time release study of siRNA from the RNA nanoparticles by Dicer processing. As a result of the siRNA release and incorporation into RISC, target Lamin A/C mRNA was cleaved as demonstrated in **Fig. 1**. These experiments prove that the RNA nanoparticles do not inhibit or limit the Dicer binding, reading,

or processing of siRNA due to their three-dimensional shapes. Additionally, in understanding Dicer processing of siRNA, several design principles of siRNA incorporation into RNA nanoparticles were created. First, the length of incorporated siRNA is very important, in that a minimum 22 nt sequence of the anti-sense strand is needed, with 24 nt showing the best processing efficiency by Dicer. Additionally, we have demonstrated that 2'-F modifications to the siRNA sense strand and removal of the 3'-UU overhang of the siRNA anti-sense results in reduced Dicer processing, but still produces siRNA product for gene knockdown. The reduced Dicer processing of the 2'-F modified siRNA is similar to that seen in studies by other groups looking at using 2'-F and 2'-OMethyl modifications on siRNA without nanoparticles.[90, 91] Collingwood *et al.* completed various modifications to both sense and anti-sense strands and overall reported reduced activities for Dicer processing.[90] However, some modifications to siRNA have demonstrated an increased potency and even the ability to incorporate into RISC without being processed by Dicer.[91, 92] As a whole, these studies advance our understanding of the role of RNA nanoparticle design to maximize the efficiency of siRNA delivery to cancer tumor cells. Additionally, these design parameters ensure RNA nanoparticles to be stable and are able to reach tumors *in vivo*. Moving forward these studies will be implemented.

RNA nanoparticles provide a stable *in vivo* delivery platform of RNAi, where target tissue specificity can be enhanced via conjugation of tumor-targeting ligands in addition to their rubber-like properties.[33, 93] Currently there are three platforms for delivery of siRNAs in clinical trials or approved by the FDA.[94] These include lipid-based nanoparticles (LNPs),[95-97] *N*-acetylaglactosamine conjugated siRNA (GalNac-siRNA),[98-100] and targeted RNAi Molecule (TRiM).[101, 102] These siRNA delivery platforms have become increasingly efficient at delivering siRNAs by now including targeting ligands. However, many of them typically accumulate in

liver and lungs. However, RNA nanoparticles have demonstrated their ability to avoid strong accumulation in the liver, lungs, and other healthy organs with only having strong accumulation in targeted tumors. Also, unlike other GalNAC-siRNA conjugates that only target hepatocytes, RNA nanoparticles are able to harbor a variety of targeting ligands to target and accumulate in a variety of cancers including colon, breast, prostate, gastric, liver and glioblastoma.[45, 46, 75, 103-105] The rubbery property, ability to deform shape under force and return to its original form upon relaxation, of RNA nanoparticles allows for a strong passive targeting effect to cancers, while being able to slip through glomerular filtration and excrete to urine through the kidneys before creating toxicity to healthy organs.[93]

Additionally, RNA nanoparticles have been used to deliver dicer-substrate siRNAs to tumor sites resulting in specific gene silencing and tumor inhibition. Cui *et al.* delivered *BRCAA1* siRNA to gastric cancer tumors in mice using the pRNA-3WJ labeled with folic acid.[44] The folate conjugated onto the nanoparticle allowed for specific binding and internalization to gastric cancer cells while specifically silencing *BRCAA1* leading to tumor growth inhibition. In a similar fashion, Lee *et al.* delivered luciferase siRNA as a proof of concept to glioblastoma tumors using folic acid targeting to the overexpression of folate receptors on the tumor model.[46] Additionally, Zhang *et al.* utilized the pRNA-3WJ platform to deliver *MED1* siRNA to breast cancer tumors in mice to overcome tamoxifen resistance.[106] The silencing of *MED1* by the RNA nanoparticles led to tumor inhibition as well as resensitization to tamoxifen, thus decreasing lung metastasis and cancer stem cell content. Xu *et al.* demonstrated the silencing of Delta-5-Desaturase (*D5D*) by pRNA-3WJ nanoparticles alongside the DLGA treatment for the colon cancer suppression.[107] Here our studies build upon this foundation of using RNA nanoparticles to deliver siRNA to cancers by examining the design properties of the substrate siRNA. Past studies have focused on

incorporating 19 nt anti-sense siRNA onto 3WJ RNA nanoparticles; however, here we proved that longer 24 nt siRNA provides an increase in Dicer processing. Additionally, careful consideration must be taken for the chemical modification of the siRNA sense strand. The proven ability of RNA nanoparticles to deliver RNAi components specifically to tumors further aids in moving RNA as the third milestone in pharmaceutics following small molecule and protein based drugs.

Materials and methods

Preparation of RNA nanoparticles harboring siRNAs

pRNA-lamin25 nanoparticles were designed with the lamin targeting siRNA extended off the 3'/5' proximate end of the phi29 pRNA. The sequence of the single stranded RNA nanoparticle is shown in **Figure S3**. Nanoparticles were constructed via bottom-up assembly through the construction of a dsDNA template including the T7 RNA polymerase promotor. RNA was transcribed *in vitro* by T7 RNA polymerase using previously described procedures in the Guo lab.[40, 108]

Different DS/*LUC2*-siRNAs were designed to study factors affecting Dicer processing. 3WJ/*LUC2*-siRNA molecular beacon was designed to study intracellular Dicer processing. The sequences are listed in **Table S1** and **Table S2**. Each short oligo strand to compose the 3WJs was prepared by solid phase synthesis using an oligo-synthesizer as described previously.[103] Molecular beacon was added to the 3WJ nanoparticles by attaching a Cy5 fluorophore to 5'-*LUC2*-antisense strand using Cy5-Phosphoramidite while BBQ650 was conjugated to 3'-3WJ-c using BBQ650-CPG column. All strands were synthesized, desalted and purified before use.

To assemble into DS- or 3WJ- nanoparticles, equal molar ratios of each component strand was mixed together in 1× TMS buffer (50 mM Tris (pH 7.6), 100 mM NaCl, 10 mM $MgCl_2$), heated to 90 °C for 5 min and slowly cooled to 37 °C on a thermocycler.

Cell culture

KB cells were cultured in RPMI-1640 medium (Life Technologies) containing 10% FBS in a 37 °C incubator under 5% CO_2 and a humidified atmosphere. HT29 cells were cultured in RPMI-1640-Folate deficient medium (Life Technologies) containing 10% FBS in a 37 °C incubator under 5% CO_2 and a humidified atmosphere.

Detection of lamin A/C siRNA knockdown by 5' RACE

100 nM pRNA-lamin25 was transfected into KB cells using FuGENE HD (Roche, Indianapolis). Cells were harvested 48 hr after transfection. Total mRNA was isolated using Poly(A)Purist™ Kit (Ambion, Austin). FirstChoice ® RLM-RACE Kit (Ambion, Austin) was used in the 5' RACE experiment or known as single-sided PCR. Using primers for the middle of the unknown sequence, mRNA is amplified in the full length therefore amplifying either the cleaved and shortened mRNA from the siRNA or amplifying the much longer uncleaved mRNA. Thus, the PCR amplification allows for easy differentiation of cleaved product. The sequences of primers used in this study are:

Lamin Gene specific 3' primer: 5'-CCAGTGAGTCCTCCAGGTCTCGAAG-3'

Lamin Gene specific 3' nested primer: 5' CCTGGCATTGTCCAGCTTGGCAGA-3'

In vitro siRNA processing using Recombinant Human Dicer enzyme

406 To examine the Dicer processing, 1 μg of RNA nanoparticles were incubated with 2 μl
407 Recombinant Human Dicer Enzyme (Genlantis) following manufacture's instruction. After 6 hr
408 incubation at 37 °C, 20% Native PAGE was used to check the product before and after the Dicer
409 processing. The gel was run in TBM buffer at 150 V for 1.5 hr, stained with Ethidium bromide
410 (EB) and imaged on a Typhoon FLA7000 (GE) for EB and Cy5 signal.
411
412 *Stability studies of 3WJ/LUC2-siRNA nanoparticles*
413 The serum and thermodynamic stability of 3WJ/*LUC2*-siRNA were studied using the
414 procedures previously described.[38, 76] In short RNA nanoparticles were incubated in 10% fetal
415 bovine serum for 0-24 hr and ran on 15% TBM polyacrylamide gel electrophoresis (PAGE). Gel
416 bands were quantified by ImageJ and plotted as Stable RNA/Total RNA. Thermodynamic
417 stability was examined by running RNA nanoparticle in temperature gradient gel electrophoresis
418 (TGGE) with a temperature gradient (20-80 °C) perpendicular to the electrophoretic current.
419 Bands were quantified by ImageJ and plotted in a similar fashion. 15% TBM PAGE was run to
420 check the integrity and purity of the nanoparticles.
421
422 *Purification of exosomes*
423 Exosomes were purified using a modified differential ultracentrifugation method as
424 previously described.[30, 56, 78] HEK293T cells obtained from ATCC were cultured in FiberCell
425 Hollow Fiber Bioreactor (C2011, 20kDa MWCO) using DMEM medium with 10% exosome
426 free FBS. Exosome-enriched media were collected every week from the bioreactor. Exosome
427 enriched media were spun down at 300 rcf for 10 min to remove cells, followed by spinning at

10,000 rcf for 30 min at 4 °C followed by 22 nm filtration to remove cell debris and micro vesicles and store at -80 °C until 500 mL accumulated.

Pre-processed media were thawed slowly at 4°C overnight, then loaded into a pre-conditioned Pall Minimate^{TM} TFF system with 100kDa MWCO capsule (OA100C12), precondition following standard operation by manufacture. 500 mL exosomes-enriched media were processed at 6 mL/min by setting pump speed at 40 mL/min. When volume reduced to ~ 5 mL, 200 mL sterile DPBS was added as a washing step and continued to run until the volume reduced to ~ 5 mL again, then collected. Two 15 mL DPBS wash steps were performed and total 30 mL wash was collected and combined with the 5 mL sample from last step.

The 35 mL post TFF media were then further purified by 100,000 rcf ultracentrifugation using a SW28 rotor (Beckman Coulter) for 90 min at 4 °C. 200 µl of 60% iodixanol (Sigma) was added to the bottom of each tube to serve as iso-osmotic cushion as previously reported.[30, 56, 78] The supernatant was carefully removed from the top and around 2 ml of the fraction close to the interface and cushion was collected.

Exosome characterization

Nanoparticle Tracking Analysis (NTA) was carried out using the Malvern NanoSight NS300 system on exosomes re-suspended in PBS at a concentration of 10 µg of protein per millilitre for analysis following published methods.[79] Three 10-second videos recorded all events for further analysis by NTA software. The Brownian motion of each particle is tracked between frames, ultimately allowing for calculation of the size through application of the Stokes–Einstein equation.

Purified exosomes were assayed for exosomal protein markers via Western blot. Exosomes were loaded onto TDX FastCast SDS PAGE gels (BioRad, Hercules, CA), ran at 100 V for 2 hours, and transferred from gel to membrane. Membranes were blocked by 5% fat-free milk at room temperature for 1 h and incubated overnight in primary antibody. Protein bands were detected with an ECL system (Pierce) after incubating in the HRP-conjugated secondary antibody for 1 h at room temperature and exposed to film for autoradiography. Primary antibodies used for western blot analysis were rabbit anti-human *TSG101* (Thermo Scientific, PA5-31260), rabbit anti-human integrin α4 (Cell Signaling, 4711S), rabbit anti-human integrin α6 (Cell Signaling, 3750S), rabbit anti-human integrin β1 (Cell Signaling, 4706S), rabbit anti-human integrin β4 (Cell Signaling, 4707S), rabbit anti-human integrin β5 (Cell Signaling, 4708S), rabbit anti-human Glypican 1 (Thermo Fisher, PA5-28055).

Loading of 3WJ/LUC2-siRNA and 3WJ/siSur into FA/EV and EGFR/EV

Exosomes (EV) (100 μg of total protein) and RNA nanoparticles harboring *LUC2* siRNA or Survivin siRNA (10 μg) were mixed in 100 μl of PBS with 10 μl of ExoFect Exosome transfection (System Biosciences), followed by a heat-shock protocol to complete the RNA nanoparticle loading. Cholesterol-modified 3WJ/FA or cholesterol-modified 3WJ/EGFR (or 3WJ) nanoparticles were incubated with RNA-loaded exosomes for decoration at an average ratio of 5000:1 (RNA:exosome) at 37 °C for 45 min, then left on ice for 1 hr to prepare the FA/EV and EGFR/EV. To purify RNA-decorated EVs, 400 μl of RNA-decorated EVs were washed with 5 ml PBS in a SW-55 tube that contained 20 μl of 60% iodixanol cushion and spun at 100,000 g for 70 min at 4 °C. All the pellets in the cushion were collected and suspended in 400 μl of sterile PBS for further use.

Binding and internalization assay of FA/Exo/Luc2-siRNA

For cell binding assays, HT29 cells were incubated with FA/EV/*LUC2*-siRNA/Cy5, 3WJ/EV/*LUC2*-siRNA/Cy5, 3WJ/FA/*LUC2*-siRNA/Cy5, 3WJ/*LUC2*-siRNA/Cy5 at 37 °C for 1 hr before Flow Cytometry. Samples were incubated at a concentration of 100 nM of the Cy5 RNA strand.

To study internalization, FA/EV/*LUC2*-siRNA/Cy5 as well as control groups including 3WJ/EV/*LUC2*-siRNA/Cy5, EV/*LUC2*-siRNA/Cy5 were incubated with HT29 cells at 37 °C for 1 hr. Samples were incubated at a concentration of 100 nM of the Cy5 RNA strand. After washing with PBS, the cells were fixed by 4% paraformaldehyde and stained by Alexa Fluor® 488 phalloidin (Invitrogen) following manufacturer's instructions and DAPI for cell nucleus staining and mounted with ProLong@ Gold Antifade Reagent (Life Technologies Corp., Carlsbad, CA). The cells were then analyzed for binding and cell entry by an Olympus FV3000 Confocal System microscope.

Intracellular imaging of siRNA processing from 3WJ via molecular beacon

To monitor intracellular Dicer processing, RNA nanoparticles (3WJ/*LUC2*-siRNA-tru beacon, 3WJ/*LUC2*-siRNA beacon, 3WJ/*LUC2*-siRNA Cy5) were loaded to exosomes respectively, further decorated by FA as described above. After incubation with HT29 cells for 0.5, 2 and 4 hr, cells were fixed and stained as described above. Samples were incubated at a concentration of 100 nM of the Cy5 RNA strand. Cy5 signal was monitored for each group at different time point by confocal microscopy under the same parameters.

Conclusions

Through the completed studies we aimed to demonstrate the efficient processing of siRNA from RNA nanoparticles for high gene silencing, while providing a comprehensive, mechanistic understanding of siRNA Dicer processing from RNA nanoparticles. As such, our pRNA-Lamin25 and 3WJ-*LUC2* beacon nanoparticles proved intercellular Dicer cleavage, RISC incorporation, and specific mRNA cleavage. Furthermore, our in-depth studies of Dicer processing of siRNA and RNA nanoparticle/siRNA conjugates proved not only that the RNA nanoparticle does not interfere in Dicer's ability to read and process siRNA sequences, but also provided design principles of incorporation of Dicer-substrate siRNA onto the nanoparticles. It can be concluded that the siRNA must be longer than 21 nt in length and allows for flexibility of chemical modifications and the inclusion of phosphate and linker nucleotides, albeit at a slight efficiency penalty. We have thus proven here, along with previous studies, RNA nanoparticles serve as a powerful platform for the efficient delivery of siRNA.

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Conflict of Interest

P.G. is the consultant of Oxford Nanopore Technologies; the cofounder of Shenzhen P&Z Bio-medical Co. Ltd, as well as cofounder of ExonanoRNA, LLC and its subsidiary Weina Biomedical LLC in Foshan. The content is solely the responsibility of the authors and does not necessarily represent the official views of NIH.

Author Contributions

Conceptualization, P.G., S.G.; Methodology, S.G., H.Y., P.G., T.J.L.; Investigation, S.G., H.Y., T.J.L.; Writing-Original Draft, H.Y., D.W.B., P.G.; Writing-Review & Editing, D.W.B., S.L., P.G.; Visualization, D.W.B., S.G., P.G.; Funding Acquisition, P.G.

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List of Figure Legends

Figure 1: Identification of the specific cleavage site of mRNA delivered by RNA nanoparticles into KB cells. A. Site specific cleavage of Lamin A/C mRNA induced by pRNA-lamin demonstrated by 5' RACE. pRNA-lamin25 was constructed by linking a siRNA sequence (underlined) with a pRNA vector via UU linkers at the sense (upper) and antisense (lower) strand. Sequences of Lamin A/C mRNA that are identical with the siRNA were underlined, and the cleavage site was marked with a star. Sequencing results shows that the PCR product contains a RNA ligator sequence (red) and part of the Lamin A/C mRNA sequence. **B.** PCR products resulting from outer primer pairs were separated in 1.2% agarose gel.

Figure 2: Construction and stability studies of 3WJ/Luc2-siRNA. A. Design of 3WJ/*LUC2*-siRNA molecular beacon (Red star: Cy5 fluorophore, Black circle: BBQ650 quencher). **B.** Assembly of 3WJ/*LUC2*-siRNA molecular beacon assayed by 15% Native PAGE (green: EtBr, Red: Cy5, Yellow: overlap). **C.** Serum stability and **D.** thermodynamic stability of 3WJ/*LUC2*-siRNA assayed by 15% Native PAGE.

Figure 3: Experimental design for intracellular imaging studies. A. Folate-Exosomes designs carrying RNA nanoparticle beacons (3WJ/*LUC2*-siRNA-truncated beacon, 3WJ/*LUC2*-siRNA beacon, 3WJ/*LUC2*-siRNA/Cy5); Red star: Cy5 fluorophore, Black circle: BBQ650 quencher. **B.** Intracellular imaging of Dicer processing of 3WJ/*LUC2*-siRNA delivered by exosomes after 0.5 hr incubation with HT29 cells. **C.** Intracellular imaging of Dicer processing of 3WJ/*LUC2*-siRNA delivered by exosomes after 2 hr incubation with HT29 cells. **D.** Intracellular imaging of

Dicer processing of 3WJ/*LUC2*-siRNA delivered by exosomes after 4 hr incubation with HT29 cells. Blue: nuclei, Green: Cytoskeleton, Red: RNA.

Figure 4: The impact of siRNA length and chemical modifications on Dicer processing. A. *In vitro* Dicer processing of DS/*LUC2*-siRNA in different length. (19-, 21-, 22-, 23-, 24- bp; p: phosphate group; S: sense strand; AS: antisense strand). **B.** and **C.** *In vitro* Dicer processing of different designs of DS/*LUC2*-siRNA. (p: phosphate group; S: sense strand; AS: antisense strand). Each sample was run as siRNA control without Dicer (-) and with Dicer (+). **D.** *In vitro* processing of siRNA from 3WJ RNA nanoparticles when incubated with cell lysates. In **A-C**, the treated siRNAs were separated in 1.2% agarose gels; and in **D**, separated in 15% Native PAGE. All siRNA cleavage was calculated by gel band quantified by Image J.

Figure 5: Summary of previous animal trials to elucidate the processing and releasing of Survivin siRNA incorporated in the RNA nanoparticles, as shown in models of non-small-cell lung,[82] breast,[30] prostate cancer.[30] RNA nanoparticles harboring Survivin siRNA were loaded into exosomes and decorated with either EGFR (epidermal growth factor) or PSMA (prostate specific membrane antigen) RNA aptamers and delivered to prostate cancer, triple negative breast cancer (TNBC), and non-small-cell lung cancer (NSCLC) tumors in mice. Repeated administration demonstrated strong tumor inhibition over control nanoparticles. The data in this figure is the summary of previously published work. Inclusion of the previously published animal data follows the journal policy of *Molecular Therapy Nucleic Acids*, with figure copyright permission from the publishers and original data from the authors Ref [30, 82].

eTOC Synopsis

SiRNA for silencing genes and treating diseases has been a popular dream but not fully realized. Peixuan Guo and colleagues report the rational design of siRNA to RNA nanoparticles for efficient delivery while avoiding endosomes. Thus providing guidelines for siRNA design on RNA nanoparticles to overcome previous roadblocks.

A.

Sequence of pRNA-Lamin25

5'-cuGGAcuuccAGAAGAAcAucuAcA-uu-(pRNA vector)-3'

3'-uu-GAccuGAAGGucuucuuGuAGAuGu-uu-(pRNA vector)-5'

mRNA sequence of lamin gene

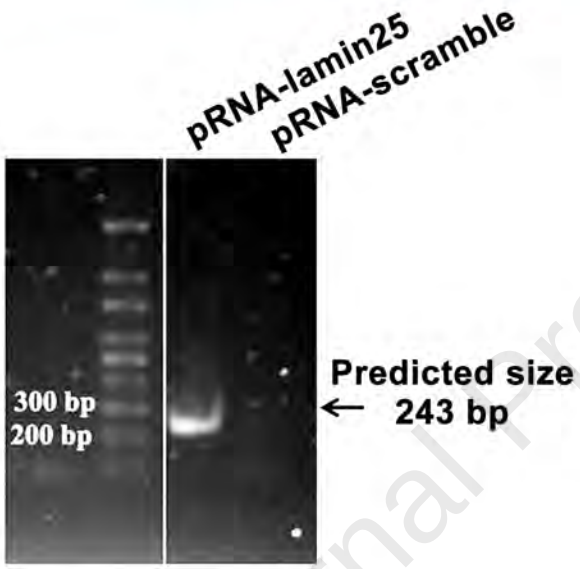
5'-...cagaccaugaaggaggaacuggacuuccagaag*aacaucuacagugag...-3'

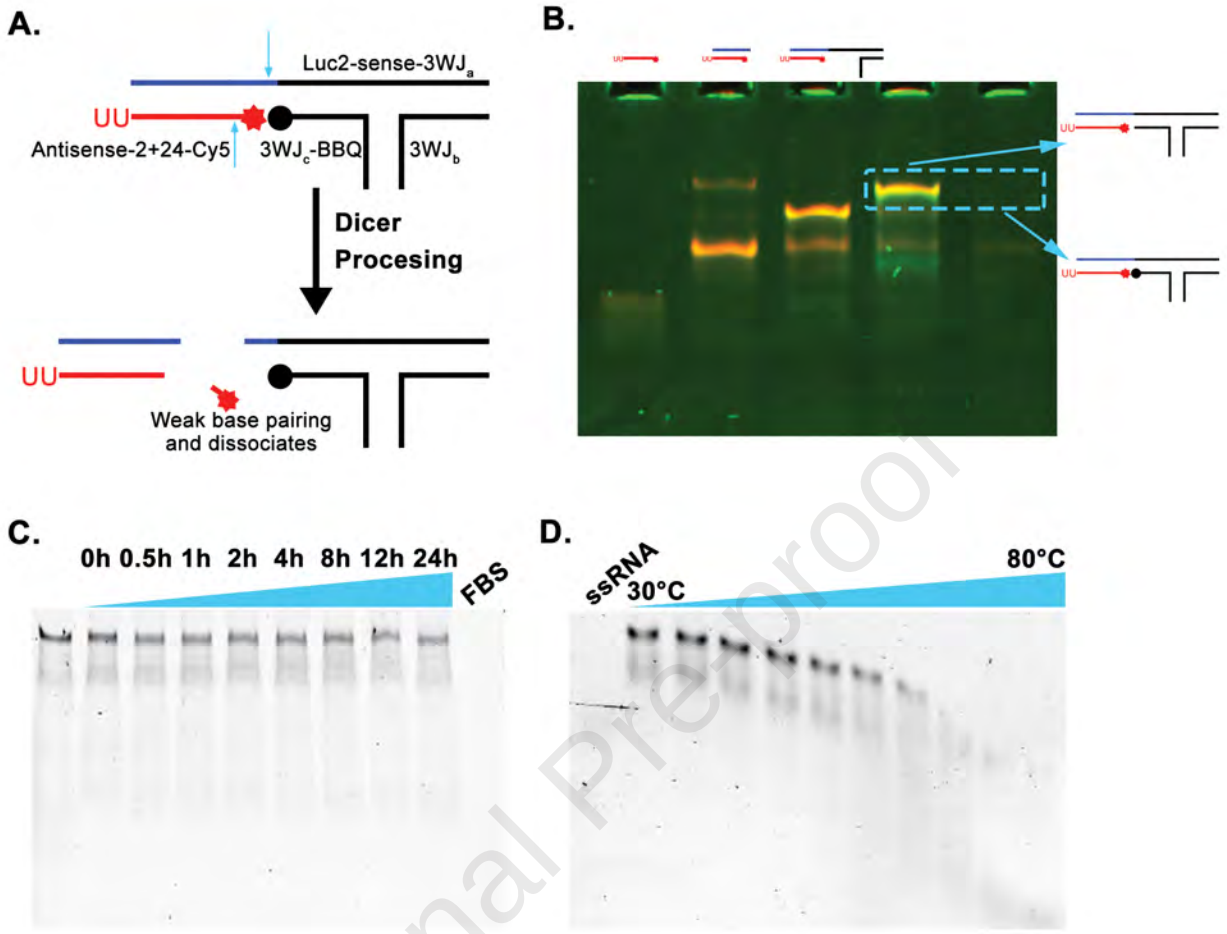
Sequence of PCR product

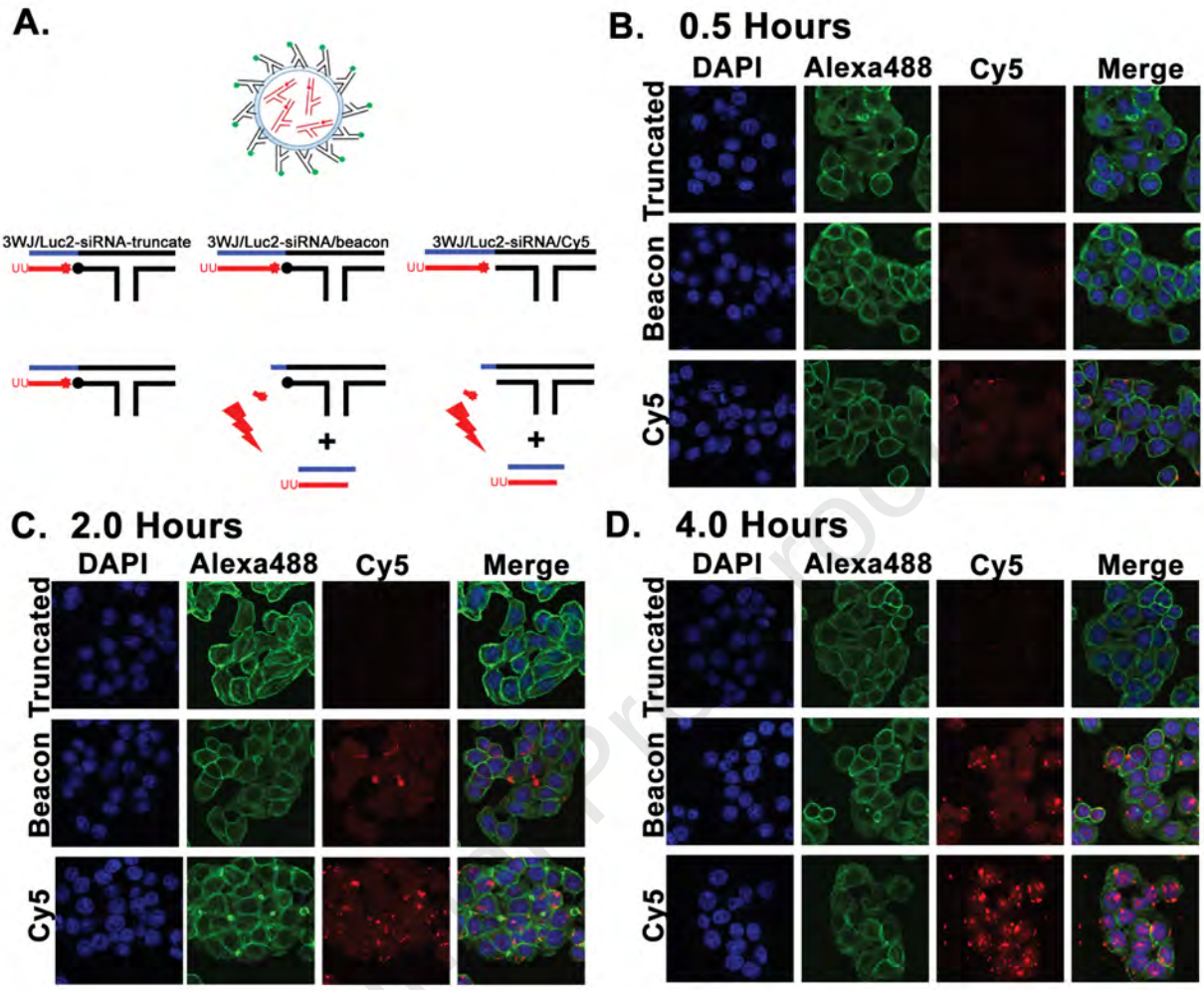
5'-NTCGCGGATCCGGAACACTGCGTTTGCTGGCTTTGATGAAAAACATCTACAGTGAGGAGCTG...-3'

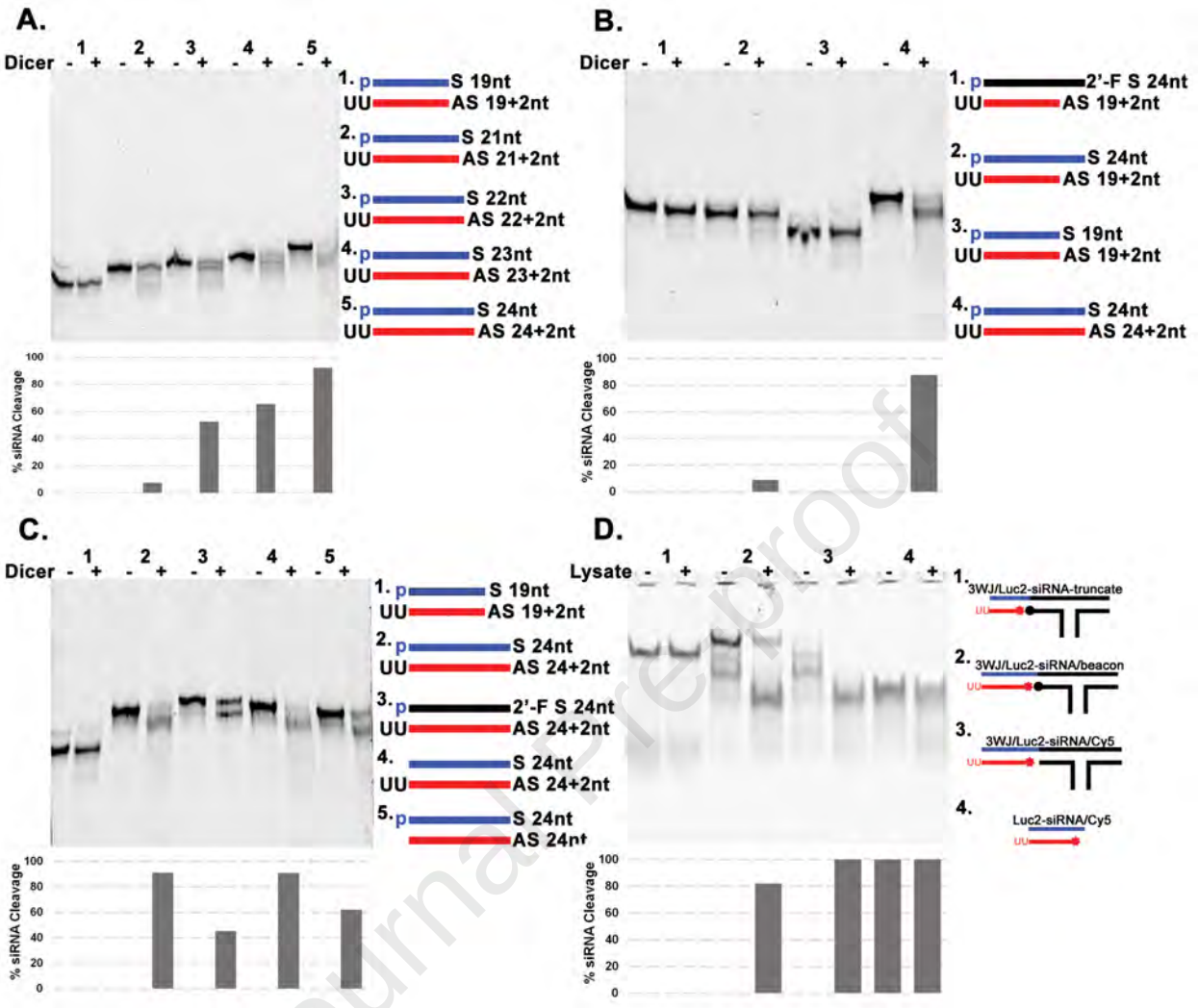
(----------RNA adaptor introduced by ligation----------)(-----mRNA sequence of lamin-----)

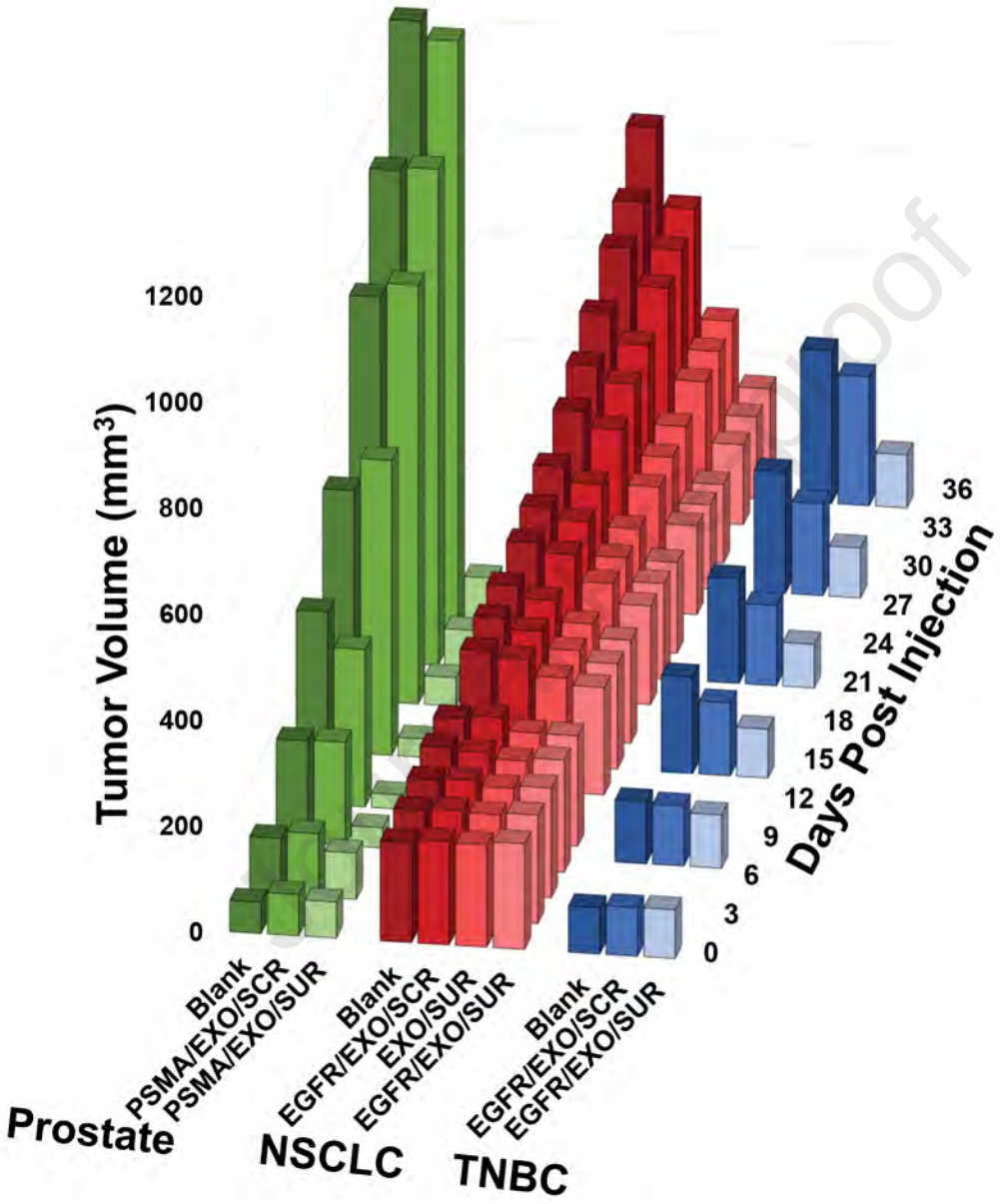
B.










Tumor Volume (mm³)
1200
1000
800
600
400
200
0
Days Post Injection
36
33
30
27
24
21
18
15
12
9
6
3
0
Blank
PSMA/EXO/SCR
PSMA/EXO/SUR
Blank
EGFR/EXO/SCR
EXO/SUR
EGFR/EXO/SUR
Blank
EGFR/EXO/SCR
EGFR/EXO/SUR
Prostate
NSCLC
TNBC