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PRINCIPAL INVESTIGATOR: Xiaoting Zhang Ph.D.

CONTRACTING ORGANIZATION: University of Cincinnati
Cincinnati, OH 45221

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E-Mail: zhangxt@ucmail.uc.edu

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14. ABSTRACT

Breast cancer is the most common type of cancer and a leading cause of death among Western women. Most breast cancers (75%) express ERα, and antiestrogens have been widely used in their treatment. However, acquired resistance and unwanted side effects in other estrogen-responsive tissue such as uterus have greatly limited their use. The objective of this study to isolate RNA aptamers that specifically target the estrogen receptor interacting NR boxes/LxxLL motifs of the MED1 protein and test their efficacy on breast cancer cell growth both in vitro and in vivo by utilizing a pRNA nanodelivery system for tissue-selective therapy. In this funding period, we have generated large amount of highly purified MED1 protein and RNA aptamer library. We further carried out and optimized SELEX experiments to select RNA aptamers that specifically bind wild type but not mutant MED1 LxxLL motifs. After 5 rounds of selections, we have now obtained several RNA aptamers for further studies. With our in vivo orthotopic xenograft experiments confirming MED1 requirements for breast tumor growth, we are now ready to further test these RNA aptamers both in vitro and in vivo.

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Introduction

Breast cancer is the most common type of cancer and a leading cause of death among Western women. Most breast cancers (75%) express ERα, and antiestrogens have been widely used in their treatment. However, acquired resistance and unwanted side effects in other estrogen-responsive tissue such as the uterus have greatly limited their use. This study is based on our recent unexpected finding that disruption of the transcriptional coactivator MED1 nuclear receptor-interacting NR boxes/LxxLL motifs in vivo specifically impaired ERα function in pubertal mammary gland development, but did not affect the development of other estrogen-responsive tissues. Significantly, MED1 is reported to be overexpressed and amplified in a high proportion of primary breast cancers and breast cancer cell lines, which has recently been confirmed in several genome-wide microarray analyses of human breast cancer patient samples. We *hypothesize* that targeting MED1 NR boxes /LxxLL motifs by aptamers will lead to tissue-selective blockage of the estrogen signaling pathway and inhibition of human breast cancer cell growth. The objective of this study is to isolate RNA aptamers that specifically bind the MED1 LxxLL motifs and test their efficacy on breast cancer cell growth both in vitro and in vivo by utilizing a pRNA nanodelivery system.

Body

To achieve our goals above, we have carried out following experiments and used the SELEX (Systematic Evolution of Ligands by Exponential Enrichment) procedure to isolate RNA aptamers that bind to MED1 NR boxes/LxxLL motifs. We have made significant advancement in these areas of the study during this first year funding period:

Generation of MED1 NR Boxes Expression Vectors

We have carried out to generate bacterial expression constructs that contain MED1 gene fragments that code for the MED1 NRBox protein or mutant derivatives. We have generated a total of 4 constructs containing MED1 NRBox-WT and NRBox-A, NRBox-B, and NRBoxAB, respectively. NRBox-WT refers to the wild type protein, which has both LXXLL motifs intact, whereas NRBox-A has LXXAA replacing the first LXXLL motif in the sequence. Similarly, NRBox-B has LXXAA replacing the second LXXLL motif, and NRBox-AB has both LXXLL motifs replaced with LXXAA sequences. To achieve this, we first used the PCR method to generate each of these wild type and mutant MED1 fragments and then ligated them individually into two types of His6-tag containing bacteria expression plasmid vectors: PET23a and PRSET. Both PET-23a and PRSET plasmids contain the His6-tag to be used for protein purification, with only difference that the His-tag for the protein produced from the PET-23a vectors is at the C-terminus and for protein from PRSET, it is at the N-terminus of the peptide. Our successful cloning of these MED1 fragments was confirmed by DNA sequencing.

Bacterial expression and Protein Purification

We then introduced these plasmids individually into the bacteria DE3 strain for protein expression. We first grew these bacteria cultures to appropriate OD and then induced with IPTG for approximately three additional hours. The cells were then lysed and the extracts were run through a HisPur Ni-NTA column (Thermo Scientific). The protein products were then

quantified via Bradford assay and visualized through SDS-PAGE followed by Coomassie Blue staining. After initial testing of the induction and purification in small scales, we expanded it into large 800ml culture in order to obtain sufficient amount of proteins for the subsequent SELEX procedure. In the end, we have been able to purify about 10mg protein for each of these constructs after repeating these experiments about 15 times and growing about more than 50 Liters of bacteria cultures. Although the protein obtained from this procedure is reasonably pure (Figure 1A), it still contains small amounts of undesired protein bands. To overcome that, we further purified the target MED1 proteins by using FPLC column separation approaches. As shown in Figure 1B, we have been able to further purify the protein with over 95% purity that is suitable for the experiments described below.

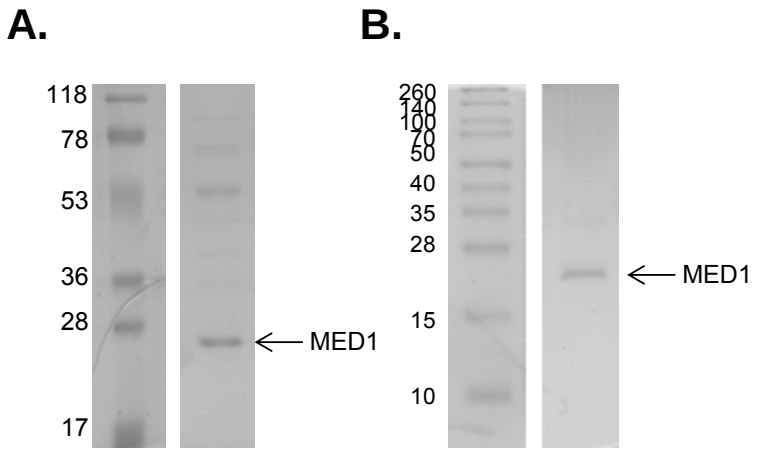

Figure 1: Purification of MED1 NR Box/LxxLL motifs protein. Purification of MED1 NR Box/LxxLL motifs protein by Nickle Column, followed by further purification using sizing column by FPLC. These data indicate that we have been able to obtain highly purified MED1 protein suitable for SELEX experiments.

RNA Library Construction

Another key element to the SELEX procedure is to generate a RNA oligonucleotide library to select RNA aptamers that bind to the MED1 NRboxes WT protein produced above. To achieve this, we have attempted to use two types of library template: library 1 is composed of 41nt random sequences flanked by about 20nt constant sequences at each end that forms an open structure, library 2 composed of 30nt random sequences flanked by 3WJ sequences of pRNA that is able to form a closed 3-way junction. We carried out RNA transcriptions using the DNA oligo libraries based on these two designs in attempt to generate sufficient RNA (1mg) for SELEX procedure (Figure 2A). We should also mention that these RNA transcriptions were carried out using 2'F modify dUTP and dCTP so the RNA oligos selected through this process can be readily applied in the future for in vivo studies because fluorination of these nucleotides has been shown to increase the stability of the resultant RNA in the blood stream.

We also tried a variety of reaction conditions to optimize the yield of RNA. Variations included using different buffer system, the amount of template used, the amount of T7 polymerase used, and whether or not IPP was added. Figure 2B shows one of such experiemnts that indicate that while the components of the buffers were largely quite similar, additional Mg-Acetate and MnCl but not IPP can enhance the production of RNA library. Ultimately, we optimized the reaction condition after many trials and were finally able to yield approximately 30-50 ug of RNA per 100µL reaction after gel isolation and purification by using the library 1

described above. However, the yield for library 2 remains extremely low. At the end, we are able to produce about 1084.9µg and 93.3µg RNA from the library 1 and 2 through numerous large scale preparations, respectively. We then decided to focus on using highly purified RNA library 1 (Figure 2C) for our SELEX experiments.

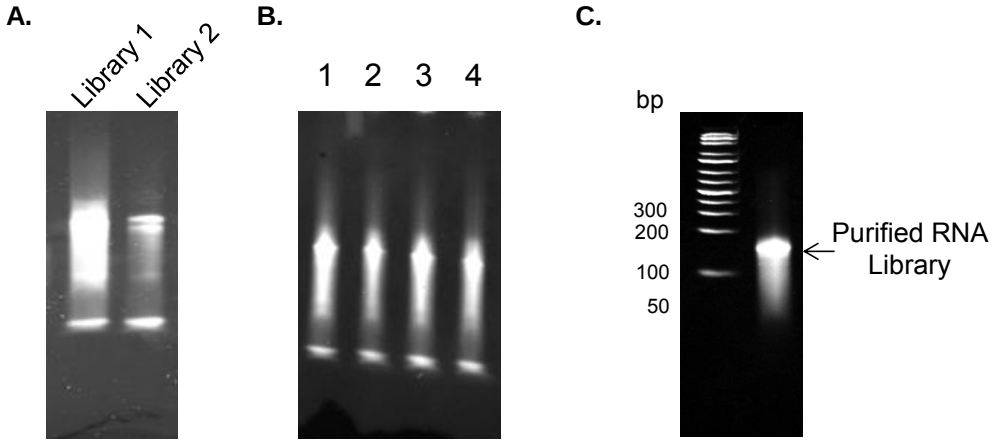

Figure 2: RNA Library Transcription. A). Urea/PAGE gel of transcribed RNA library 1 and 2. B) Urea/PAGE gel of transcribed RNA library 1 under the following condition (1. Mg-Acetate and MnCl, no IPP; 2. No Mg-Acetate and MnCl, no IPP; 3. Mg-Acetate and MnCl, with IPP; 4. No Mg-Acetate and MnCl, with IPP). C) Urea/PAGE gel of transcribed RNA library 1 after gel-purification indicating high-quality RNA library ready for the SELEX procedure.

SELEX Experiments

As mentioned above, we have obtained approximately a total of 10mg of NRBox-WT protein and 1mg of RNA library 1 and were therefore ready for the SELEX experiment. To begin, we first mixed 1 mg of NRBox-WT protein and RNA library 1 generated with Ni-NTA Beads, respectively, and rotated for two hours. The protein that bound to the beads was then combined with the RNA solution pre-cleared with Ni-NTA beads and incubated for approximately 30 minutes. The bound RNA was then eluted using an imidazole-containing buffer and then extracted followed by ethanol precipitation. To further

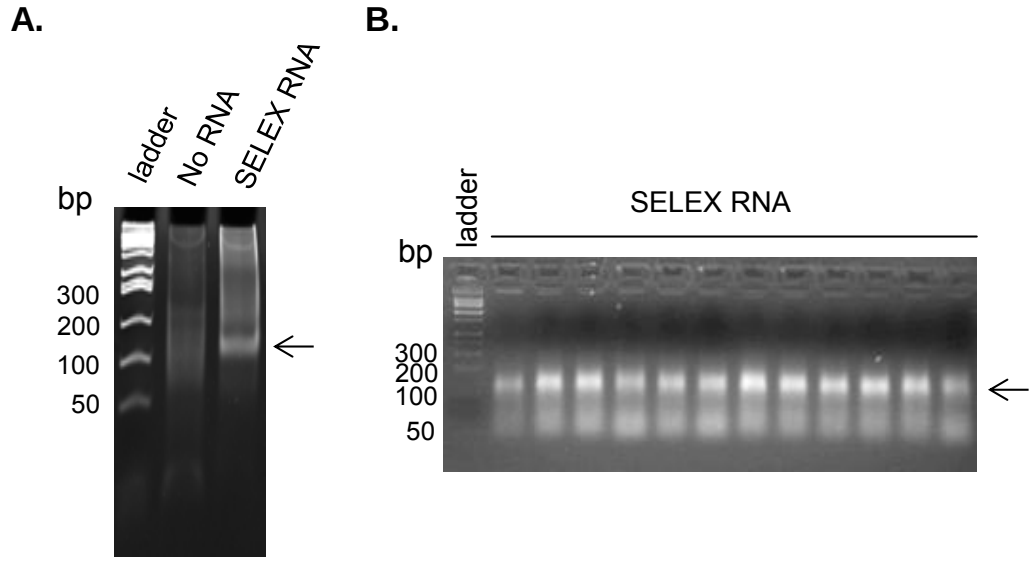

Figure 3: RT-PCR amplification of MED1 NR box/LxxLL motifs - Binding RNAs. MED1 LxxLL motifs-Bound RNAs recovered from SELEX process were subjected to RT-PCR amplification. The PCR products were then run on A) PAGE/Urea gel or B) 3% agarose gel. The data indicate that our RT-PCR amplifications were highly specific (A). These products were then run on agarose gel (B) to be purified by gel isolation kits (Qiagen) for next round of RNA library construction.

amplify this isolated RNA products for next round of SELEX procedure, we then performed reverse transcription experiments to create a cDNA library. To maximize the yield and minimize the non-specific PCR amplification, we utilized different reverse transcription systems and tried different amounts of primer for each amplification steps. At the end, we decided to utilize AMV reverse-transcriptase from Biolabs with approximately 10µg RNA per 20uL reaction to generate highly specific first strand cDNA (Figure 3A), followed by two to three rounds of PCR to generate sufficient amount of cDNA template for RNA library construction. The first round of PCR had 15 cycles while the second and third usually will last 35 cycles. In order to isolate our DNA segment, we also experimented with several different types of gel. First we used a vertical native 8% PAGE gel. This gel provided optimum visualization of our products; however, elution of the DNA from excised gel pieces was difficult and resulted in a very low yield. The next attempted gel was a 3% Synergel, which, after several attempts, never provided visualization of a distinct band. Finally, we found that running a high percentage of agarose gel (3%) with high amount ethidium bromide provided relatively good visualization (Figure 3B) and could easily be excised and eluted from the gel by using Qiagen PCR purification system. To date, we have performed 5 rounds of SELEX procedure and the recovery rate of the RNA bound to our target protein from the SELEX experiment has increased 256 fold (Table I). We then cloned the resulting RT-PCR fragments of the final round into Topo TA cloning vector. We have now sequenced 30 clones and obtained the DNA sequence coding for these RNA aptamers. We are therefore ready to test their functions both in vitro and in vivo as proposed in Aim 2.

	RNA Library	Protein	Bound RNA	Recovery Rate
Round 1	499.9ug	1mg	65ng	0.013%
Round 2	131ug	1mg	35ng	0.026%
Round 3	500.42ug	1mg	310ng	0.062%
Round 4	119ug	1mg	1075ng	0.90%
Round 5	300ug	300ug	2990ng	3.32%

Table I. RNA Recovery Rate after each round of SELEX procedure. The table shows the amount of RNA library, protein used in each round of SELEX procedure and the bound RNA recovered. The data indicate a 256 fold increase of RNA recovery rate after 5 round of SELEX.

Orthotopic tumor xenograft studies.

As planned, we have also initiated the in vivo mouse mammary gland orthotopic xenograft experiments in preparation for treatment with Aptamers isolated above. In this experiment, we used BT474 cells integrated with luciferase reporter gene for the purpose of in vivo imaging. In addition to that, we have introduced MED1 shRNA expression vector under the control of a Tet-ON promoter into these cells (Figure 4A) so we can induce the expression of MED1 shRNA expression by using Doxycycline to determine the efficacy of knocking down MED1 on tumor growth (Figure 4B). We first injected BT474-luc(tet-on-MED1shRNA) cells orthotopically into mouse mammary gland and then treated with Doxycycline through water feed. We found that treatment of doxycycline significantly decreased the tumor growth of these cells when compared to the control group (Figure 4C and D). These results indicate that MED1 plays essential roles in the growth of BT474 tumors in vivo and that our orthotopic tumor

xenograft system is ready. Therefore, we are on track and ready for the experiments using RNA aptamers isolated above in this model to examine their roles for tumor growth in vivo.

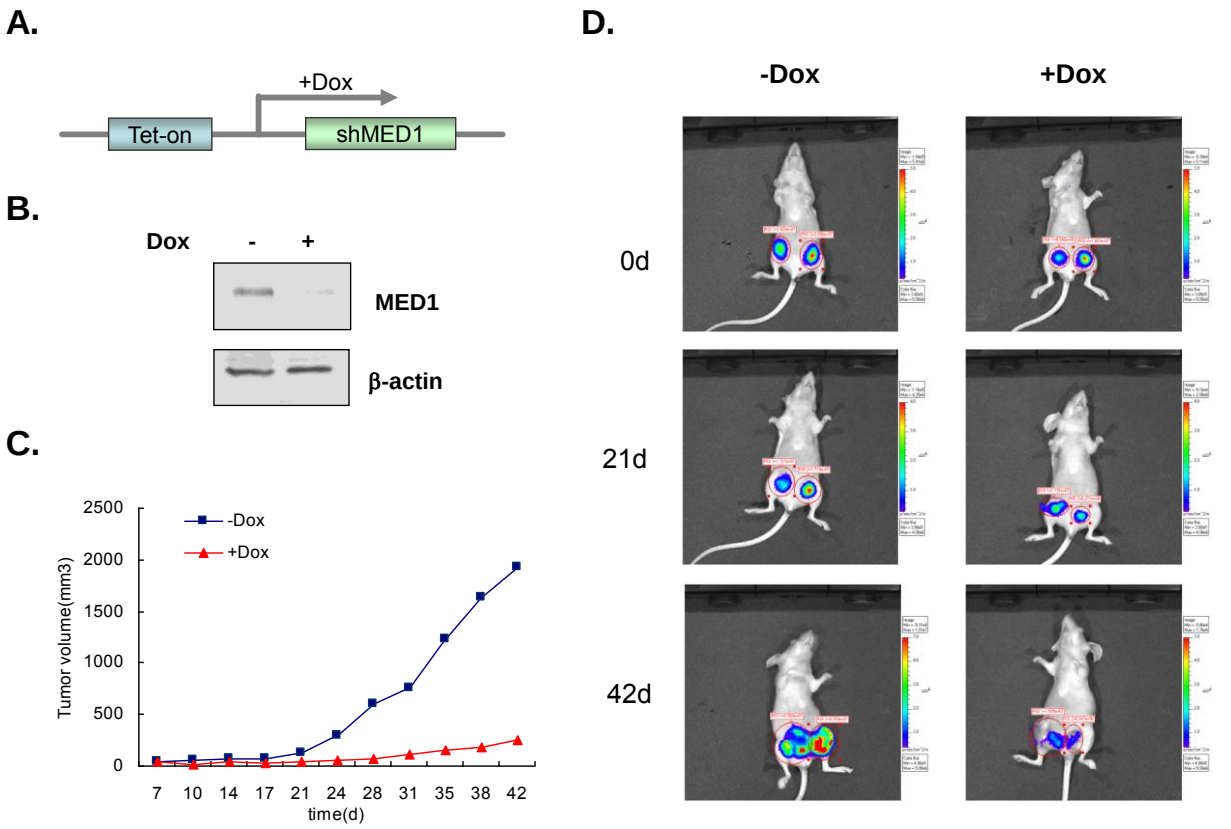

Figure 4. MED1 is required for breast cancer cell growth in vivo in orthotopic xenograft mouse model. A). Diagram shows construction of Tet-On inducible MED1 shRNA expression construct. B). Western blot analyses indicate successful knockdown of MED1 protein expression in BT474-luc cells after Dox induction. C). Tumor growth were measured in control and Dox treated groups by calculating tumor volume and plotted. D). Representative images of BT474-luc tumors in control and Dox treated groups at indicated time by using Caliper Xenogen in vivo imaging system after injection of luciferin substrate. These data indicate the requirement of MED1 for breast cancer growth in vivo and our successful establishment of in vivo orthotopic xenograft mouse model to test above selected RNA aptamers against MED1.

Key Research Accomplishments

--- Cloned NRBox-WT, NRBox-A, NRBox-B, and NRBoxAB fragments into pET23 and pRSET vectors for expression as His6-tagged proteins;
--- Optimized the protein expression induction conditions for each of above constructs and obtained large quantities (10mg) of proteins for each NRBox-WT, NRBox-A, NRBox-B, and NRBoxAB fragments;
--- These Proteins were further purified by FPLC to reach over 95% of purity;
--- Synthesized two RNA libraries for the SELEX procedure and optimized the conditions for their construction;
--- Generated and purified sufficient large amount of RNAs (>1mg) of RNA library 1 for the SELEX procedure;
--- Carried out 5 rounds of SELEX procedure to isolate RNA aptamers that bind NRBox-WT protein;
--- The resulting RNA aptamers isolated has been reverse transcribed, amplified and sequenced for further studies;
--- Carried out Orthotopic tumor xenograft studies that further validate the essential role of MED1 for tumor growth in vivo and prepares for our further experiments to test the function of the RNA aptamers isolated above on breast cancer growth in vivo.

Reportable Outcomes

In this reporting period, we have two manuscripts (Attached as Appendices for this report) now in-press and two abstracts accepted by international conferences:

Papers:
Germer K, Pi M, Guo P and **Zhang X.** Conjugation of RNA aptamer to pRNA nanoparticles for RNA-based therapy. (2012) RNA Nanotechnology and Therapeutics, CRC Press. (Corresponding Author, In-press)

Germer K and **Zhang X.** RNA aptamer and its diagnostic and therapeutic applications. (*Book Chapter*) (2012) RNA Nanotechnology and Therapeutics, CRC Press (Corresponding Author, In-press)

Abstracts:
Jiang P, Zhang D, Xu, Q, Meeks, J, Zhang, L and Zhang X. Transcriptional Cofactor MED1 in Mammary Stem/Progenitor Cell Determination and Breast Cancer. *(Platform Presentation)* Zing Cancer Conference 2011--- *From Carcinogenesis to Cancer Therapy*, Nov. 9-13, 2011, Occidental Grand Xcaret, Mexico

Zhang D, Jiang P, Xu, Q, and Zhang X. Key Roles of Transcriptional Coactivator MED1 in Breast Cancer Cell Growth. *(Session Chair and Speaker, Stem cell and Breast Cancer)*, BIT's 4th Annual World Cancer Congress-Breast Cancer Conference 2011, Nov. 15-18, 2011, Guangzhou, China

Conclusion

In conclusion, during this first-year funding period, we have successfully generated His6-tagged expression constructs for MED1 NRBox WT and corresponding mutants. We have expressed these proteins and obtained large quantities (10mg each) of these proteins with very high purity. We have also constructed and purified large quantities of RNA library 1 after optimizing the reaction conditions. We then carried out SELEX experiments using the RNA library and proteins purified. We have now already carried out 5 rounds of the SELEX procedures and sequenced the RNA aptamers isolated. Therefore, we are in a very good position to perform the next round of experiments to test the functions of these RNA aptamers isolated on breast cancer cell growth in vitro and in vivo. Importantly, we have already carried out in vivo mammary gland orthotopic tumor xenograft studies, which confirmed the essential role of MED1 for tumor growth. Therefore, this system is readily available in our laboratory to test the function of these aptamers. In addition to these, we have also written two book chapters on RNA aptamers now in-press for the Book RNA Nanotechnology and Therapeutics by CRC Press, and presented our work at two international conferences.

References

N/A

Appendices

In-Press Manuscript 1: Conjugation of RNA aptamer to RNA nanoparticles for targeted drug delivery.

In-Press Manuscript 2: RNA aptamers and their diagnostic and therapeutic applications.

[RNA Nanotechnology and Therapeutics, CRC Press, In-Press]

Conjugation of RNA aptamer to RNA nanoparticles for targeted drug delivery

Katherine Germer[1], Fengmei Pi[2], Peixuan Guo[2] and Xiaoting Zhang[1]

[1]Department of Cancer and Cell Biology, Vontz Center for Molecular Studies, University of Cincinnati College of Medicine, Cincinnati, OH 45267; [2] School of Pharmacy and Nanobiotechnology Center, University of Kentucky, Lexington, KY 40536

Key Words: RNA nanotechnology, RNA Aptamer, pRNA, Drug Delivery

Corresponding Author's Mailing Address:

Xiaoting Zhang
Department of Cancer and Cell Biology
3125 Eden Avenue
Cincinnati, OH 45267
Tel: 513-558-3017
Fax: 513-558-4454
Email: zhangxt@ucmail.uc.edu

Abstract

Recent isolation of RNA aptamers with high affinity and specificity rivaling that of antibodies makes them promising tools for targeted delivery of therapeutics. However, a major challenge that remains is the systemic and intracellular delivery of RNA aptamers to the desired target cells due to their sub-nano scale structures. Conjugation of RNA aptamers to pRNA nanoparticles, which was pioneered by Dr. Guo's group, has paved a path for their use in targeted drug delivery. These pRNA nanoparticles can be fabricated as a biocompatible material by many different means, including dimer and hexamer formations, or most recently, through a stable formation of three-way junction pRNA motifs. The approaches and key factors for successful conjugation of RNA aptamers to the pRNA nanoparticles for targeted delivery, while maintaining their functionality, are further discussed in this chapter. Finally, the current advancements in RNA aptamer-conjugated pRNA nanoparticle applications for the treatment of cancer and viral infections are summarized.

1. Introduction

RNA-based therapy has become a promising avenue for the treatment of many human diseases. Therapeutic potentials of RNAs, including ribozymes, short hairpin RNA (shRNA), siRNA, miRNA, antisense oligonucleotides (AS OGNs), and RNA aptamers, have long been extensively studied (Guo, 2010; Keefe et al., 2010; Levy-Nissenbaum et al., 2008; Que-Gewirth and Sullenger, 2007; Yan and Levy, 2009). A major challenge that remains is the systemic and intracellular delivery of these moieties (siRNA, ribozyme, etc) to the desired target cells. In this regard, the pRNA nanoparticle delivery system pioneered by Dr. Guo, combined with recent advancement in RNA aptamers, provides an ideal method for nanoscale delivery suitable for in vivo targeted delivery (Guo, 2010).

pRNA nanoparticles are a new RNA based nanoparticle drug delivery system, which can be designed and constructed by phi29 pRNA through dimer formation, hexamer formation, or using its stable three way junction domain as a scaffold. Since pRNA nanoparticles are composed of all RNA, it is the most natural choice for the delivery of RNA therapeutics because using an all RNA delivery system will also allow all the advantages of RNAs as therapeutic agents to be retained. One key obstacle for RNA-based therapy is that RNA is susceptible to quick degradation in the blood stream during in vivo delivery. To overcome that, Dr. Guo's laboratory has recently developed highly stable and RNAse-resistant pRNA through elaborate design of RNA sequences and chemical modifications such as 2'-deoxy-2'-fluoro (2'-F) modification at the ribose rings of C and U (Liu et al., 2011), (Shu et al., 2011).

RNA Aptamers are RNA oligonucleotides capable of binding to specific targets with high affinity and specificity. RNA aptamers have numerous advantages for targeted drug delivery when compared to DNA aptamers, protein aptamers, and antibodies (Guo, 2010; Keefe et al., 2010; Que-Gewirth and Sullenger, 2007; Thiel and Giangrande, 2010). Compared to their peptide and antibody counterparts, RNA aptamers are much easier to synthesize in large quantities with defined structure and stoichiometry. Furthermore, RNA aptamers are generally considered to be more thermodynamically stable than peptides or antibodies. Although RNA aptamers function similarly to antibodies, they are known to have low or no immunogenicity when compared to other macromolecules such as proteins/antibodies. Furthermore, recent studies has found that RNA aptamers can be further chemically modified (e.g. 2'deoxy, 2'F, 2'NH3, 2'OMe) to achieve high stability and evade RNAase shearing even in the blood stream. Moreover, the single-stranded nature of RNA aptamers not only allows them to form unique tertiary structures for tighter and more specific binding to the target, but also makes them smaller

in size and thus easier to enter into cells than other types of aptamers. Thus, conjugation of RNA aptamers to delicately designed pRNA nanoparticles can assist in the delivery of therapeutics harboring RNA nanoparticles to specific cell organelles to maximize the therapeutic effects while minimizing the toxicity of the drug delivery system.

2. Structure of RNA nanoparticles

The concept of RNA nanotechnology has been proposed for more than one decade. Most RNA molecules are single stranded nucleotides, which can adopt very complex three-dimensional structures. Therefore, RNA is an ideal biocompatible material which is suitable for construction to nanometer scale for drug delivery, especially for oligonucleotides-based drug delivery.

2.1 RNA nanoparticle formed by dimerization of phi29 pRNA

Phi29 pRNA is a 117 nucleotide bacteriophage phi29 encoded packaging RNA (pRNA) discovered by Dr.Guo in 1987 (Guo et al., 1987). pRNA monomer plays an essential role in packaging DNA into procapsid by forming a hexamer ring to drive the DNA packaging motor of bacteriophage 29, which is about 11nm in size. The primary structure of wild type pRNA is described in Figure 1(Liu et al., 2011). pRNA has two functional domains that can fold independently: DNA translocation domain and prohead binding domain. The DNA translocation domain is composed of a 3'/5' double helix loop while the prohead binding domain is composed of left- and right- hand loops. If we name the right hand loop with uppercase letters A, B, C and name the left hand loop with lowercase letters a, b, c denoting different loop sequences, the RNA sequence for A, B, C are complementary to sequence a, b, c respectively. pRNA dimer nanoparticles can be formed through the complementary hand in hand loop interactions between

pRNA monomer Ab and Ba as described in Figure 2 (Shu et al., 2004) . The pRNA dimer nanoparticle has been reported to have a particle size of about 25nm (Chen et al., 2000), which allows it to be employed as one nanoparticle carrier for gene drug delivery since the small size can escape from being engulfed by the reticuloendothelial system, and can be used for repeated and long term gene drug delivery.

2.2 RNA nanoparticle composed by using pRNA hexamer as scaffold

pRNA dimers are building blocks for pRNA hexamer formation, which has been proved by Chen et al (Chen et al., 2000), through hand in hand interaction of two complementary pRNA can form dimer, tetramer and hexamer. A schematic drawing for the pRNA hexmaer formation is described in Figure 3. The six pieces of pRNA in pRNA hexamer nanoparticles could provide six positions to conjugate therapeutic molecules such as siRNA, ribozyme, therapeutic RNA/DNA aptamer, or diagnostic RNA/DNA aptamer for drug delivery.

2.3 RNA nanoparticles based on three way junction motif

A third strategy for constructing pRNA nanoparticle is based on a thermodynamically stable RNA three way junction (3WJ) motif (Shu et al., 2011). pRNA has two functional domains that can fold independently: DNA translocation domain and prohead binding domain. The two domains are connected by a three way junction motif, as described in Figure 4. The 3WJ domain of pRNA was demonstrated to be very stable, which can retain its folding even in 8M Urea or at very diluted concentration. By conjugating the RNA therapeutic molecules such as siRNA or RNA aptamers to the 3WJ motif, they can self assemble to form RNA nanoparticles, which potentially can be used for targeted RNA therapeutic delivery in vivo.

3. Conjugate RNA aptamer to RNA nanaoparticles for targeted drug delivery

The concept of a targeted drug delivery system was proposed by Paul Ehrlich in 1902, in which he first called the hypothetical drug a “magic bullet” (Ehrlich, 1957). In general, targeted drug delivery requires the targeted drug delivery system (TDDS) to selectively deliver therapeutics to diseased regions, independent of the method of its administration. TDDS can be classified into three grades from the aspect of the region it reaches: the first grade targeting system refers to delivering the drug to a targeted organ or tissue, the second grade targeting system refers to delivering the drug to specific cells, and the third grade targeting system refers to delivering therapeutic molecules to specific locations inside the cell.

TDDS can also be divided into three types based on the pattern of targeting: passive, active and physical. There has been intensive research on passive targeting drug delivery systems, such as liposomes, nanoemulsions, microcapsules and polymeric nanospheres in the last several decades. Passive targeting relies on the natural distribution pattern of the drug delivery system, as the drug carriers can be ingested by macrophages of the reticuloendohelial system and then transferred primarily to the liver and spleen. However, it is difficult to deliver drugs to other organs with the passive targeting mechanism, because the in vivo distribution of the passive targeting drug carriers is greatly impacted by its particle size and the surface property of nanoparticles. As a general rule, when the particle size is larger than 7um, it will be retained by the smallest lung blood capillaries through mechanical filtration; when the particle size is smaller than 7um, it is ingested by macrophages in the liver and spleen; carriers of particles between 200nm to 400nm are usually collected in liver and rapidly cleaned up by the liver. Active targeting preparation instead utilizes modified drug carriers as a “bullet” to directionally concentrate drugs to the target area for enhanced efficacy. These modifications include PEGlyzation of the nanoparticles to conceal the particle from macrophages, conjugation with

special ligands or antibodies, which can interact with the target cell receptor, as well as other approaches. Physical and chemical targeting preparations utilize physical or chemical properties to help navigate preparations to specific targeting locations. For example, magnetic targeted drug delivery incorporates magnetic material into the drug preparation, and the preparation will then be concentrated to the specific target area under guidance of an externally applied magnetic field, whereas thermal or PH targeting drug delivery utilizes thermal or PH sensitive material to deliver the therapeutics to specific macro-environments by changes in temperature or PH.

3.1 RNA Aptamers and SELEX

RNA Aptamers are RNA oligonucleotides that bind to a specific target with high affinity and specificity, similarly to antibody interaction with antigens. RNA aptamer isolation was initially developed in two separate laboratories by Turek and Gold, and by Ellington and Szostak (Ellington and Szostak, 1990; Tuerk and Gold, 1990) through a process that eventually became known as Systematic Evolution of Ligands by EXponential enrichment, or SELEX. To begin the SELEX process, a library of randomized pools of RNA will first be synthesized. Generally, these RNA oligonucleotides are designed with a random sequence of nucleotides of approximately 20-80 nucleotides in the center region that is flanked on either side by a constant sequence. This library of oligonucleotides will then be exposed to the target of interest, which could be small molecules, proteins, cells, or even organisms (Dua et al., 2011; Keefe et al., 2010; Levy-Nissenbaum et al., 2008; Thiel and Giangrande, 2010). Those do not bind to the target are washed away and discarded, whereas those do bind are isolated and amplified through reverse transcription and PCR to generate a corresponding DNA library. The DNA library is then subjected to RNA transcription, and the resulting RNA library will then be exposed again to the target of interest for another round of SELEX process. This process is usually repeated about 5-

15 times, and the aptamers obtained could often reach a high pico-molar to low nano-molar range of dissociation constants (kd's) with the target (Dua et al., 2011; Yan and Levy, 2009).

Through this basic SELEX process, and some recently developed variations of this process such as Cell-SELEX, Cross-over SELEX and Tissue-SELEX, a good number of RNA aptamers have been isolated with the capability of binding numerous specific targets (Dua et al., 2011; Levy-Nissenbaum et al., 2008; Yan and Levy, 2009). Significantly, many of these targets are cell surface markers of various human diseases, which has led to the application of these RNA aptamers for targeted delivery of RNA therapeutics, especially those based on RNA interferences (RNAi): small interference RNA (siRNA), short-hairpin RNA (shRNA) or microRNA(miRNA).

3.2 Approaches to conjugate aptamer to RNA nanoparticle

The key step in the construction of RNA aptamer-conjugated nanoparticles is to design the global structure according to the physical and chemical properties of the RNA nanoparticles. If the RNA aptamer has been selected with a known sequence, it can be conjugated into the RNA nanoparticle structure before in vitro transcription or chemical synthesis of RNA. Dr. Guo and his colleagues have successfully conjugated malachite green (MG) aptamer to RNA nanoparticles characterized by a three-way junction (3WJ) pRNA motif. The in vitro experiment indicated that the aptamer is still functional after conjugation into 3WJ-pRNA nanoparticles (Figure 5) (Shu et al., 2011). The sequence for the MG aptamer nanoparticle was rationally designed with sequences of three pieces of 3WJ-pRNA motif. These three pieces of RNA strands were synthesized in vitro by transcription from DNA template with T7 RNA polymerase and the RNA nanoparticles were then self-assembled when the three RNA pieces were mixed in equal molar ratios. If there is currently no known RNA aptamer available for the desired

applications, another approach for conjugation of aptamers to RNA nanoparticle is to conjugate random sequences to a defined RNA nanoparticle structure. SELEX experiments as described above will then be carried out with this library of random sequence baring RNA nanoparticles against target peptide, protein or cell. The DNA library with random sequences is transcribed into RNA library, and then partition techniques such as nitrocellulose partition, Capillary Electrophoresis partition, etc. can be utilized to separate the bound and unbound RNA. The bound RNA nanoparticles are further eluted out and used as a template for reverse transcription and PCR for the next round of SELEX experiment.

3.3 Key factors for conjugating aptamer to RNA nanoparticles

When designing the aptamer-conjugated RNA nanoparticles, one key factor that needs to be considered is that the aptamer should be conjugated to the outsphere site of RNA nanoparticles, thus the aptamer targeting delivery functionality can be achieved. Another key factor is to ensure that the aptamer will still fold correctly after conjugation to RNA nanoparticle nucleotides. If the aptamer has a double strand helix RNA end in its structure, we can connect both ends of aptamer to the open helix ends of RNA nanoparticles. If the aptamer has a single strand RNA loop end in its structure, then we can link only one end of the aptamer to the open ends of RNA nanoparticle carriers. For example, when designing anti-gp120 aptamer conjugated pRNA nanoparticles, J. Zhou et.al designed two different chimeric pRNA-anti-gp120 aptamer constructs. In one structure, both the double strand helix ends of anti-gp120 aptamer were linked to the pRNA end bases of 23-97(pRNA-A1-D3); in another structure the anti-gp120 aptamer was directly appended to the 5'-end of pRNA(pRNA-A1-D4) (Figure 6) (Zhou et al., 2011). In vitro studies demonstrated that both pRNA-aptamer chimeras can specifically bind to and be

internalized into cells expressing HIV gp120 with the dissociation constant (Kd) for the pRNA-A1-D3 being about 48nM and for the pRNA-A1-D4 was 79nM.

4. Application Status of RNA aptamer conjugated pRNA nanoparticles

Since pRNA nanoparticles are composed of all RNA, conjugating RNA aptamers to pRNA to form a targeted delivery system will allow all the advantages of RNAs as therapeutic agents to be retained. We have discribed the structure and synthesis of both pRNA and the aptamers, and discussed the approaches and key factors in the designing of RNA aptamer conjugated pRNA. Another key obstacle in applying RNA aptamer-pRNA system for targeted therapy is that RNA is not stable and susceptible to quick degradation in vivo in blood stream. Recently, Dr. Guo's Lab found that 2'-F modification of pRNAs are both chemically and metabolically stable in vivo in animals. Importantly, they have shown that the pRNA's function and biologic activity stays intact, despite this 2'-F modification. To date, this pRNA nanoparticle delivery system has been used to conjugate CD4 aptamers and anti-GP120 aptamers, and was tested in anti-cancer and viral infection therapies.

Dr. Guo's laboratory has used the pRNA dimer nanoparticle to specifically deliver siRNA against the pro-survival gene called survivin to CD4 positive cells. This is accomplished by replacing the 3'/5' double helix loop of the pRNA sequence with the survivin-silencing siRNA and by conjugating an anti-CD4 aptamer to the pRNA. They found this dimer is able to specifically target CD4 positive lymphocytes to silence the target gene expression and reduce cell viability (Guo et al., 2005). Most recently, Dr. Guo's laboratory has discovered that the three way junction (3WJ) of pRNA discussed above is the most stable structure found among 25 3WJ motifs obtained from different biological systems (Shu et al., 2011). They have shown that

each arm of the 3WJ-pRNA can carry the above mentioned CD4 receptor binding RNA aptamer, siRNA, or ribozyme and bring them into target cells both in vitro and in vivo. Importantly, they have further gone on to show that the 2'-F RNAse resistant form of 3WJ-pRNA also retains its folding and can carry these incorporated functional moities to target cells both in vitro and in vivo.

Additionally, anti-gp120 aptamers has also been conjugated with pRNA system by Dr. Rossi's group in their research against the Human Immunodeficiency Virus (HIV-1) infections (Zhou et al., 2008; Zhou and Rossi, 2011; Zhou et al., 2011; Zhou et al., 2009). The HIV-1 virus express a surface protein called glycoprotein gp120 that recognizes the CD4 cell receptor on the host's cells and initiates the membrane fusion that leads to subsequent delivery of viral RNA and enzymes. Once infected by HIV-1, these cells will then also express gp120 on their cell surface. Zhou et al has previously generated gp120 aptamer chimeras with siRNA targeting the HIV-1 tat/rev gene region and found that these chimeras can be specifically internalized into cells expressing gp120 to silence the expression of target gene. Most recently, Dr. Rossi's group further used the pRNA system developed by Dr. Guo to generate dual functional RNA nanoparticle chemiras with anti-gp120 aptamers, as described above, and achieved both cell type specific delivery and targeted inhibition of viral replications (Zhou et al., 2011).

5. Conclusions and Future Perspective

The high affinity and specificity of RNA aptamers rivaling those of antibodies makes them a promising tool for targeted delivery of therapeutics. As discussed in this chapter, conjugating RNA aptamers to pRNA nanoparticles for targeted thereapy has shown great promise in the treatment of cancer and viral infections. In addition to the above-mentioned

pRNA nanodelivery system, there have also been developments of other approaches for delivering therapuetics using RNA aptamers for disease treatments. With their many advantages as a key component of RNA nanotechnology, including the small size, high stability, multi-conjugation capability and especially their non-immunogenic nature, RNA aptamers will no doubt find more applications in the targeted therapy arena, especially with more and more RNA aptamers isolated against an ever-increasing repertoire of disease targets. With strong interest and further development of RNA nanotechnology and recent approval of RNA as therapeutics by FDA, we should also expect a bright future for RNA aptamers not only as a delivery tool for targeted therapy but also beyond.

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Figure Legends

Figure 1. Primary sequence and structure of wild type pRNA. Figure is produced with permission from Ref (Liu et al. 2011), © American Chemical Society.

Figure 2. pRNA dimer formed through hand in hand complementary loop interactions. Figure is produced with permission from Ref (Guo et al. 2010), © Nature Publishing Group.

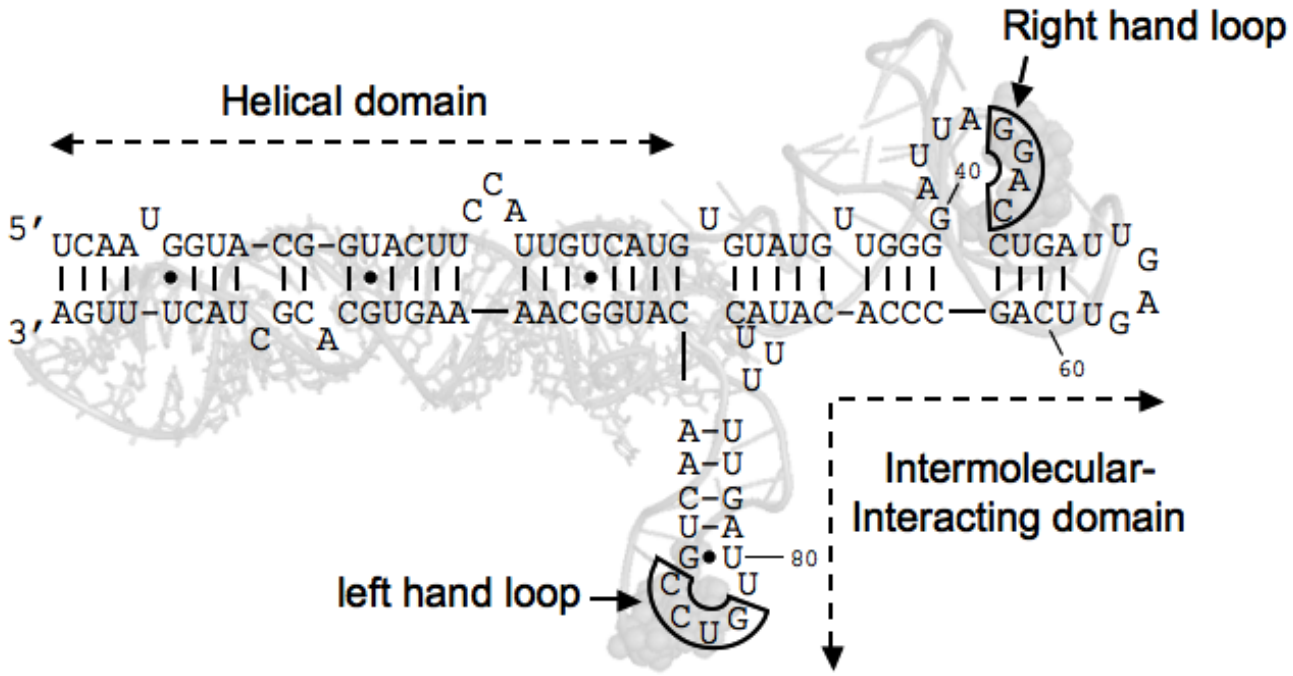
Figure 3. Schematic drawing of pRNA hexamer through complementary loop loop interaction with 6 pieces of pRNA monomer. Figure is produced with permission from Ref(Chen et al.2000), © American Society for Biochemistry and Molecular Biology.

Figure 4. Three way junction motif of pRNA, which can be used for RNA nanoparticles construction. Figure is produced with permission from Ref (Shu et al. 2011), © Nature Publishing Group.

Figure 5. Diagram of RNA nanoparticle harboring malachite green (MG) aptamer, survivin siRNA and folate-DNA/RNA sequence for targeting delivery, using 3WJ-pRNA as scaffolds. Figure is produced with permission from Ref (Shu et al. 2011) © Nature Publishing Group.

Figure 6. Schematic of pRNA nanoparticle harboring anti-HIV gp120 aptamer. (A) pRNA–A1-D3, the aptamer sequence was inserted into the 3'/5' double helical domain(23nt

fragment) and loop domain(97nt fragment). (B) pRNA-A1-D4, the aptamer sequence was directly appended to the 5'end of pRNA 5'/3' double stranded helical domain. **Figure is produced with permission from Ref (Zhou et al. 2011) © Elsevier Inc.**


Right hand loop
Helical domain
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3′ AGUU-UCAU GC CGUGAA—AACGGUAC CAUAC-ACCC—GACU G A
A-U
A-U
C-G
U-A
G•U
left hand loop
Intermolecular-
Interacting domain
40
60
80

Figure 1

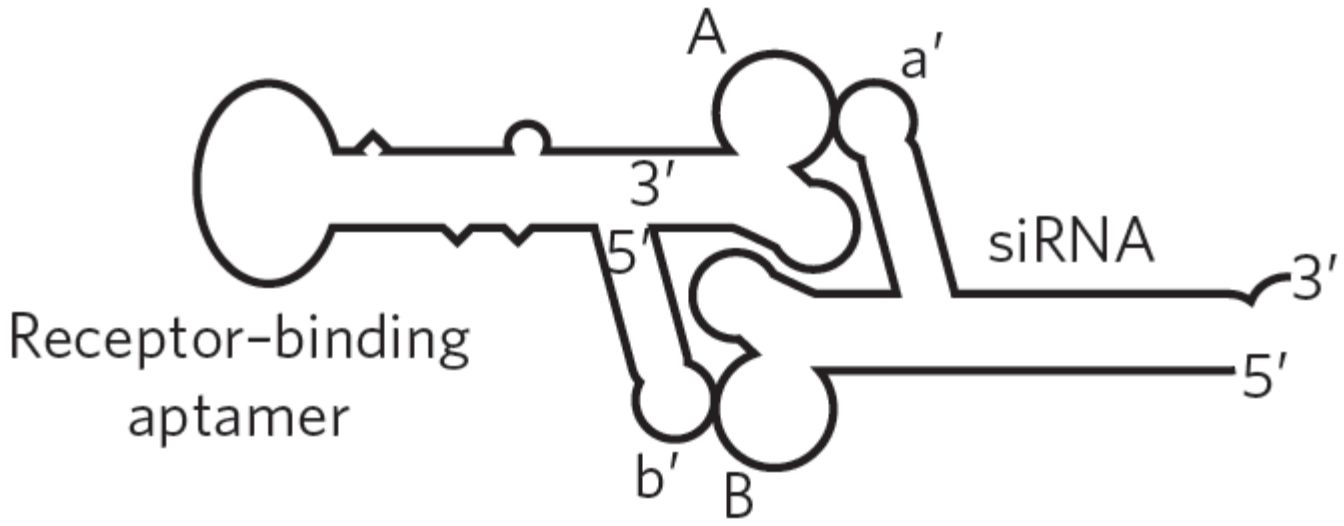

Figure 2

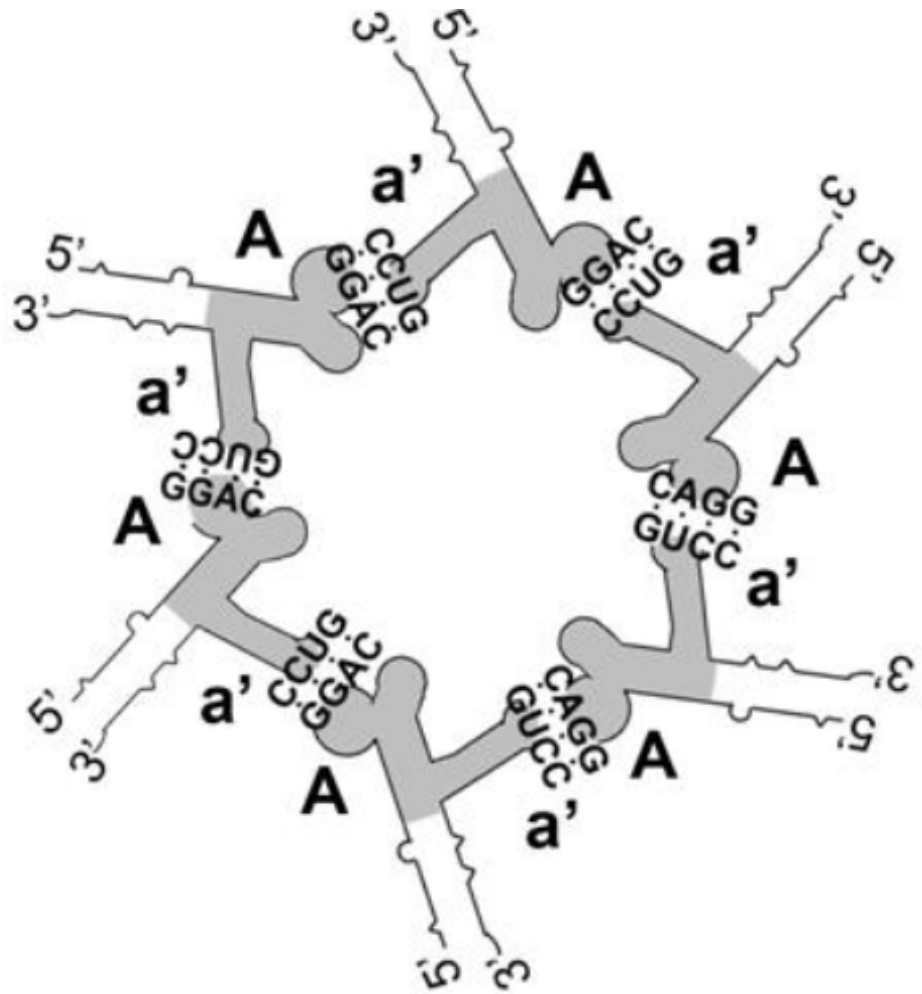
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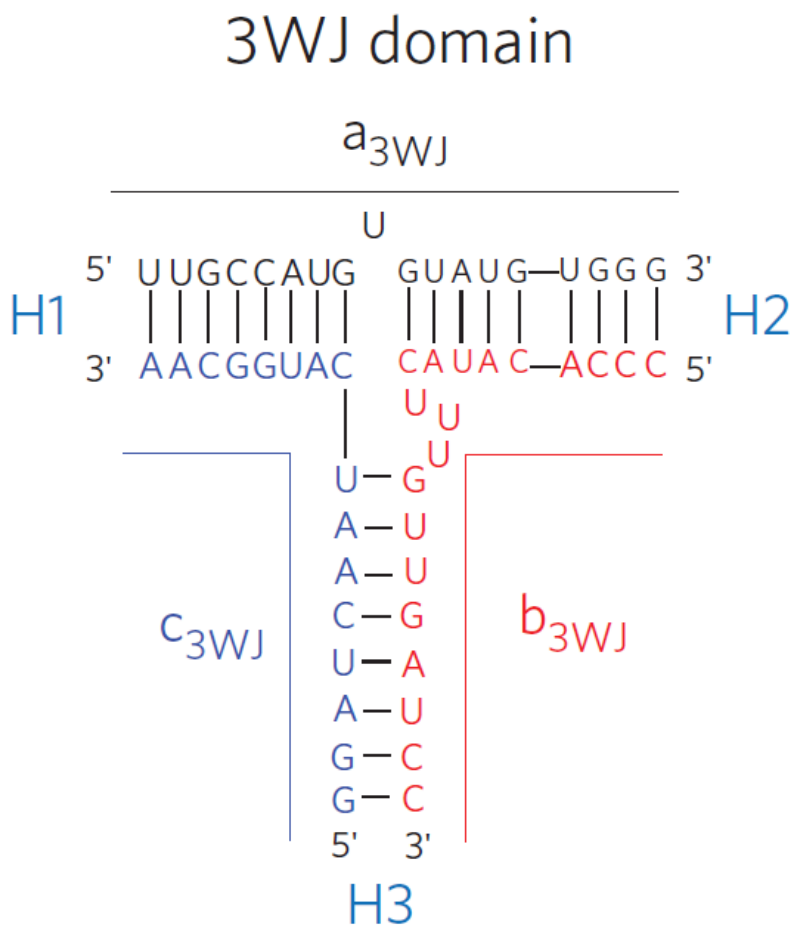
Figure 3


3WJ domain
a3WJ
U
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H1
H2
3' AACGGUAC CAUAC—ACCC 5'
U
U
U
U—G
A—U
A—U
C—G
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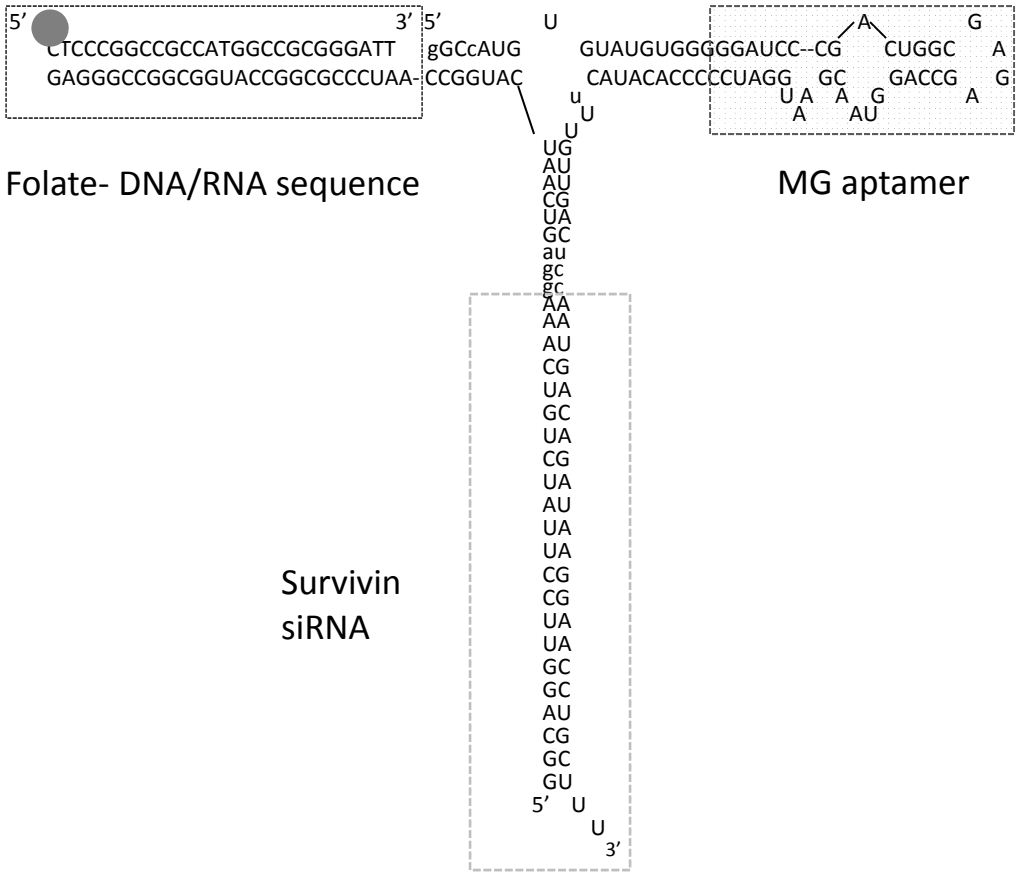
G—C
5' 3'
c3WJ
b3WJ
H3

Figure 4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3’
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5’
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CAUACACCCCCUAGG
A
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CUGGC
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GC
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AU
CG
GC
GU
5’
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Folate- DNA/RNA sequence
MG aptamer
Survivin
siRNA

Figure 5

A. pRNA-A1-D3

B. pRNA-A1-D4

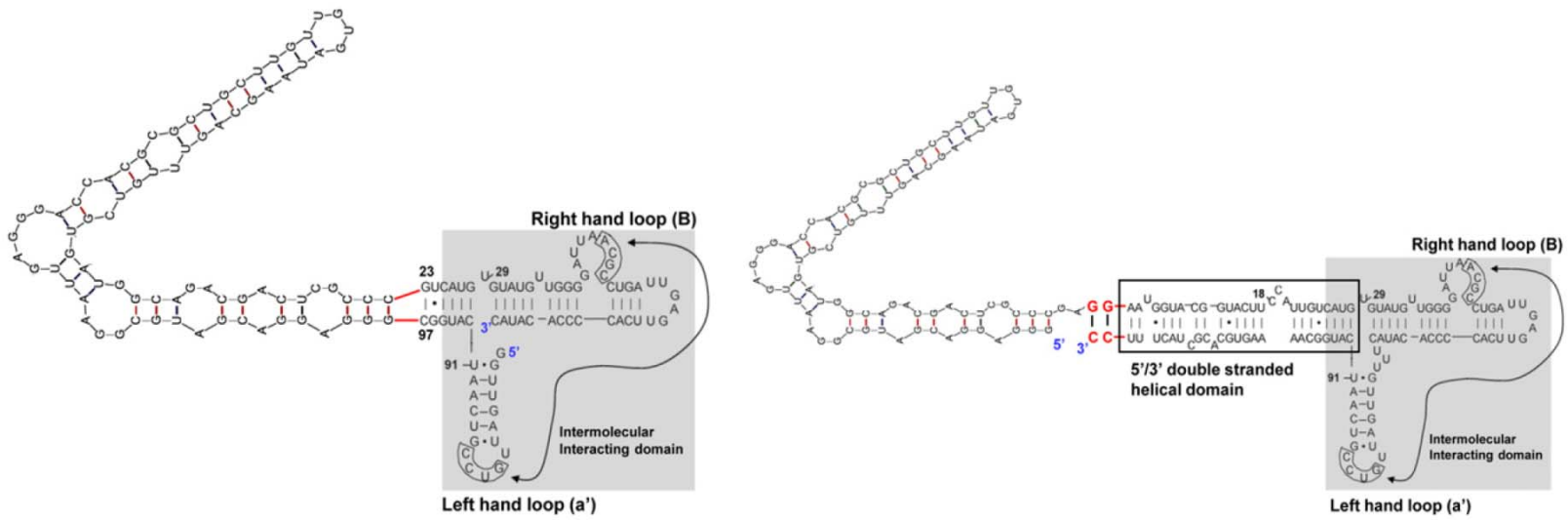

Figure 6

[RNA Nanotechnology and Therapeutics, CRC Press, In-Press]

RNA Aptamers and Their Therapeutic and Diagnostic Applications

Katherine Germer and Xiaoting Zhang

Department of Cancer and Cell Biology, Vontz Center for Molecular Studies, University of Cincinnati College of Medicine, OH 45267

Key Words: RNA nanotechnology, RNA Aptamer, SELEX, RNA Therapy, Drug Delivery

Corresponding Author's Mailing Address:

Xiaoting Zhang
Department of Cancer and Cell Biology
3125 Eden Avenue
Cincinnati, OH 45267
Tel: 513-558-3017
Fax: 513-558-4454
Email: zhangxt@ucmail.uc.edu

Abstract

RNA Aptamers refer to RNA oligonulceotides that are capable of binding to specific targets with high affinity and specificity. Through a process called Systematic Evolution of Ligands by EXponential enrichment (SELEX), a number of RNA aptamers have been identified against various targets including organic compounds, nucleotides, proteins and even whole cells and organisms. RNA aptamers have proven to be of high therapeutic and diagnostic value with recent FDA approval of the first aptamer drug and additional ones in the clinical pipelines. It has also been found to be a particularly useful tool for cell-type specific delivery of other RNA therapeutics like siRNA. All these establish RNA aptamers as one of the pivotal tools of the emerging RNA nanotechnology field in the fight against human diseases including cancer, viral infections and other diseases. This chapter summarizes the current advancement in the identification of RNA aptamers and also provides some examples of their therapeutic and diagnostic applications.

1. Introduction

RNA Aptamers are defined as RNA oligonucleotides that bind to a specific target with high affinity and specificity, similarly to how an antibody binds to an antigen. Isolation of aptamers from randomized pools of RNA by using a method called Systematic Evolution of Ligands by EXponential enrichment (SELEX) was first developed by Gold and Turek, and by Ellington and Szostak (Ellington and Szostak, 1990; Tuerk and Gold, 1990). These RNA molecules were termed as "aptamers," with etymology stemming from the Greek word *aptus*, which means "to fit" (Ellington and Szostak, 1990; Gold et al., 2010). To date, various aptamers have been successfully selected against different targets and have begun to show promise as

diagnostic, prognostic and therapeutic tools in a wide-range of applications including the treatment for human diseases such as cancer, viral infection and macular degeneration (Dua et al., 2011; Jayasena, 1999; Keefe et al., 2010; Levy-Nissenbaum et al., 2008; Que-Gewirth and Sullenger, 2007; Soontornworajit and Wang, 2011; Thiel and Giangrande, 2010; Yan and Levy, 2009).

Although this section focuses on the development and applications of RNA aptamers, it is important to note that aptamers can also be made of DNA and protein as well. However, there are numerous advantages to RNA aptamers as a pivotal tool of RNA nanotechnology when compared to DNA aptamers, protein aptamers, and antibodies (Guo, 2010; Keefe et al., 2010; Que-Gewirth and Sullenger, 2007; Thiel and Giangrande, 2010). First, although RNA aptamers function similarly to antibodies, they are known to have low or no immunogenicity when compared to other macromolecules such as proteins. A second advantage to RNA aptamers is that when compared to their peptide and antibody counterparts, they are easier to synthesize in large quantities in a controlled manner, and achieve defined structure and stoichiometry. Furthermore, nucleic acids such as RNAs are generally considered to be more thermodynamically stable than peptides or antibodies. Importantly, RNA aptamers can be further chemically modified (e.g. 2'deoxy, 2'F, 2'NH3, 2'OMe), which has been found to greatly improve their stability in the blood stream and resistance to RNAase shearing. Moreover, RNA aptamers are single-stranded in nature, which allows for a unique tertiary structure and leads to tighter and more specific binding. The single-stranded composition of RNA aptamers also makes them smaller in size and easier to enter into cells than DNA aptamers with of the same length in nucleotides. Importantly, this size-advantage and easy conjugation feature of RNA

aptamers also aid their ability to carry additional ligands for specific targeting or therapeutic agents for intracellular drug delivery.

2. SELEX: Systematic Evolution of Ligands by EXponential enrichment

Aptamers are chemically synthesized and selected for their high affinity and specificity for a certain target through the SELEX process (Ellington and Szostak, 1990; Gold et al., 2010; Tuerk and Gold, 1990). Generally speaking, an RNA aptamer is about 56-120 nucleotides long and is comprised of a variable region and a constant region. The variable portion of the aptamer is located in the center and ranges from 20-80 nucleotides in length. Constant nucleotide sequences can be found on both sides of the variable region (the 5' and 3' ends) with about 18-20 nucleotides in length. To date, through traditional basic SELEX and variations of this approach such as Cell-SELEX, Cross-Over SELEX and Tissue-SELEX (Figure 1), a good number of RNA aptamers have been isolated with the capability of binding numerous targets, including small molecules, proteins, cells, and even organisms (Dua et al., 2011; Keefe et al., 2010; Levy-Nissenbaum et al., 2008; Thiel and Giangrande, 2010).

2.1 The Basic SELEX Procedure

When initiating a SELEX procedure to isolate RNA aptamers, a library of RNA oligonucleotides with a complexity upwards of 10^{14} is first generated wherein the sequence of nucleotides in the center region is randomly generated and the flanking segments are constant, as described above (Dua et al., 2011; Ellington and Szostak, 1990; Gold et al., 2010; Levy-Nissenbaum et al., 2008; Tuerk and Gold, 1990; Yan and Levy, 2009). These oligonucleotides are then exposed to the intended target of interest under the desired conditions. A subset of the

oligos will bind to the target, and are therefore potentially the desired aptamer. They are first partitioned from those that do not bind; reverse transcribed, amplified using PCR and further transcribe to generate a new pool of RNA oligonucleotides. Subsequently, this pool of binding oligonucleotides is exposed to the target for a second time, and once again the binding oligonucleotides are isolated and amplified. This sequence is repeated, usually 5-15 times, in order to isolate the aptamers that have the best binding properties. To ensure that the aptamer binds *only* the desired target, oligos that bind the non-desired target are often removed from the pool of aptamers through a process called "negative selection," which uses targets that are similar, but not identical, in structure. In this fashion, the aptamer that binds most specifically and sensitively to the desired target can be discovered and amplified. Typically, aptamers selected through the SELEX process have dissociation constants (kd's) with the target ranging from high pico-molar to low nano-molar.

When done manually, the SELEX procedure can take more than a month to complete. Cox *et al.* recognized the potential for improving the speed of the procedure by automation. By coupling use of a robot and specially designed software, they were able to complete upwards of 10 rounds of SELEX per day (Cox et al., 1998). This not only made it possible to complete a SELEX experiment in a matter of days, but also improved the consistency of results.

2.2 Cell-SELEX

There are also different variations of the SELEX procedure that can be done; one of which is called Cell-SELEX(Dua et al., 2011; Levy-Nissenbaum et al., 2008; Yan and Levy, 2009). The fundamental difference of this procedure from traditional SELEX is that whole living cells are used as targets. Cell-SELEX has been most widely used to isolate RNA aptamers

against known or novel cell surface markers of human diseases for therapy and biomarker discovery. Cell-SELEX offers the advantage of being able to create aptamers against a desired cell type, even if the cell's markers are not known. All cells are known to express certain protein markers on their surface. In a disease state such as cancer, the levels of some these markers can increase, additional modifications may be added, and completely new cell markers can appear on the cell's surface, all of which can be used to differentiate these diseased cells from their normal state. Through this approach, RNA aptamers have been isolated against cell surface markers such as T-cell acute lymphoblastic lymphoma, glioblastoma, and small cell lung cancer, among others.

Cell-SELEX, however, is not without its drawbacks. The cells can be damaged and even undergo cell death during the procedure to separate the bound aptamers from the unbound. These dead cells bind aptamers non-specifically and therefore supply a pool of aptamers with decreased specificity for the desired protein target. Currently, work is being done to separate the cells that are live from those which have died or been damaged by using the techniques such as high-speed fluorescence-activated sorting (FACS) to separate these two populations of cells. Adding to the problem of non-specific binding is the fact that there are multiple cell markers on the cells. Because of this, aptamers may bind to the other cell membrane proteins and therefore still pass through the SELEX process. As such, it becomes very important to do the negative selection steps, as described above and below, in order to filter these aptamers out of the pool. This ultimately requires more rounds of SELEX to be carried out and thus decrease the efficiency of the process (Dua et al., 2011).

2.3 Cross-over and Tissue-SELEX

In light of the above multiple cell markers issue in Cell-SELEX, Cross-over SELEX has recently been developed for situations in which cell markers are already known for the disease process of interest. In this method, the initial rounds of SELEX are carried out using whole cells as targets as in Cell-SELEX, while later rounds are accomplished using purified cell marker protein targets. In this way, the investigator gains the advantages of both processes and yet diminishes the disadvantage of interference from other proteins (Dua et al., 2011; Levy-Nissenbaum et al., 2008; Yan and Levy, 2009).

Another variation on SELEX is called Tissue-SELEX. In this process, researchers are able to, for example, take a section of a tumor tissue and isolate aptamers that may bind different components of the tumor, including the extracellular matrix, cellular membranes and intracellular components (Dua et al., 2011). This process is accomplished by exposing diseased tissue section slides to the pool of aptamers. The healthy tissues are also exposed to serve as a control so the investigator can identify aptamers that bind solely to the diseased tissue. Remarkably, this tissue-SELEX strategy has recently been expanded to in vivo applications by using tumor-bearing mice to identify RNA aptmaers with the ability to recognize cell markers for diseases such as hepatic colon cancer metastases (Mi et al., 2010).

3. Thearpeutic and Diagnostic Applications of RNA Aptamers

Through the above mentioned various SELEX approaches, dozens of RNA aptamers have been isolated against mostly cell surface markers, but also for intra- and extracellular components of key signaling pathways (Table I) (Keefe et al., 2010; Levy-Nissenbaum et al., 2008; Que-Gewirth and Sullenger, 2007; Thiel and Giangrande, 2010). The high binding affinity and specificity of RNA aptamers, among and other characteristics, make them highly attractive

for therapeutic and diagnostic applications to target these markers or signaling pathways. In fact, this area of research has recently gained huge momentum with FDA's approval of the first RNA aptamer-based therapeutics for clinical use. Here, we will summarize the current advancements and provide several examples of applying RNA aptamers for targeted therapy, as well as their diagnostic applications in disease diagnosis, imaging, and new biomarker discovery.

3.1 Therapeutic Applications of RNA Aptamers

Therapeutic potentials of RNAs, including ribozymes, short hairpin RNA (shRNA), siRNA, miRNA, antisense oligonucleotides (AS OGNs) and RNA aptamers, has long been extensively studied (Guo, 2010; Keefe et al., 2010; Levy-Nissenbaum et al., 2008; Que-Gewirth and Sullenger, 2007; Yan and Levy, 2009). RNA aptamers have unique advantages because, in addition to their intracellular targeting capability, they can directly bind to extracellular targets to inhibit, and in some cases activate their functions, whereas other RNA-based therapeutics must first enter the cell to carry out their functions. In addition, by taking advantage of their ability to bind cell surface proteins, RNA aptamers have also been found to be particularly powerful tools to deliver a variety of therapeutic agents such as small molecules, peptides, and especially RNA-based therapeutics into specific cell types for the treatment of human diseases. In this section, we will provide several examples for recent advances using RNA aptamers as thereaputic agents and as cell-specific delivery tools for RNA-based therapy.

3.1.1 RNA Aptamers as therapeutic agents

With the advancement of RNA nanotechnology and development of RNA aptamers suitable for systematic delivery, RNA aptamers have becoming attractive thereaputic agents

against many targets, especially those in the ocular compartments, blood stream and cell surface proteins (Keefe et al., 2010; Que-Gewirth and Sullenger, 2007). In 2004, Pegaptanib (Macugen, Eyetech Pharmaceutics/Pfizer), an aptamer against Vascular Endothelial Growth Factor (VEGF), become the first RNA aptamer approved by US Food and Drug Administration for therapeutic use against age-related macular degeneration (AMD) (Ng and Adamis, 2006). Currently, there are also several other RNA aptamers under clinical and preclinical trials for the treatment of diseases such as diabetes and cancer. Here, we will briefly introduce Pegaptanib and also include an update on current efforts to identify and characterize RNA aptamers against the epidermal growth factor receptor (EGFR) family members that are often overexpressed in cancers as examples for future therapeutic applications of RNA aptamers.

3.1.1.1 Vascular Endothelial Growth Factor

Vascular Endothelial Growth Factor (VEGF) is a protein that plays essential roles in both physiologic and pathologic angiogenesis (Ferrara, 2004; Keck et al., 1989; Leung et al., 1989; Senger et al., 1983). VEGF acts to promote angiogenesis through binding to its receptors (receptor tyrosine kinsases VEGFR1 and VEGFR2) to activate its downstream signaling pathways. Through alternative splicing, the VEGF gene expresses 4 major isoforms of 121, 165, 189 and 206 amino acids, respectively. $VEGF_{165}$ isoform has been found to be solely responsible for the abhorrent neovascularization in age-related macular degeneration (AMD) and diabetic macular edema (DME) (Ng and Adamis, 2006). Taking advantage of that, researchers have carried out studies to isolate the RNA aptamer that specifically targets the $VEGF_{165}$ isoform (Green et al., 1995; Jellinek et al., 1994; Ruckman et al., 1998). In these studies, they identified several anti-VEGF aptamers with very high affinity and specificity for the $VEGF_{165}$ isoform.

Further modifications were extensively examined to increase its stability in serum by fluorination, methylation, and addition of a 3'-3'-linked deoxythmidine terminal cap as well as a 5' polyethylene glycol moiety (Ng and Adamis, 2006). Pre-clinical experiments identified one of these aptamers that has the highest biological activity in inhibiting $VEGF_{165}$'s functions as an endothelial mitogen and vascular permeability enhancer through a series of *in vitro* and *in vivo* experiments. Importantly, this aptamer also exhibited no toxicity and very long half-life in pharmacokinetics studies with one study showing biologically active aptamers still remains in eye 28 days post single intravitereous injection in monkeys (Ng et al., 2006). With its great success in preclinical studies, this aptamer was eventually developed into a drug that would undergo clinical trials for treatment of both AMD and DME. The resultant drug was named "Pegaptanib" and has completed Federal Drug Administration (FDA) trials to become available for treatment of AMD in 2004.

3.1.1.2 Epidermal Growth Factor Receptor Family

The epidermal growth factor receptor (EGFR) family consists of four closely related cell membrane receptor tyrosine kinases: EGFR (ErbB-1), HER2/c-neu (ErbB-2), Her 3 (ErbB-3) and Her 4 (ErbB-4) (Zhang et al., 2007). These EGFR family members can form homodimer and heterodimer to activate their kinase activities and downstream signaling cascades that can be facilitated by ligands such as EGF, TGF-α, heregulin. It has been found that mutations affecting the expression or activity of EGFR family proteins are often associated with the development of a wide variety of types of cancers including breast cancer, glioblastoma and lung cancer, among others (Yarden and Sliwkowski, 2001). Importantly, blocking their aberrant activation, by monoclonal antibodies (mAb) that target extracelluar dimerization or ligand binding domain or

by small molecules that target intracellular kinase domain, could significantly inhibit tumor growth and sensitize the tumors to traditional chemotherapy and radiotherapy (O'Rourke et al., 1998; Slamon et al., 2001). Owing to the above mentioned advantages of RNA as nanotherapeutics, not surprisingly, efforts have been taken by several laboratories to identify RNA aptamers that can specifically bind and block the activation of these EGFR family members.

A30 binding to the oligomeric state of extracellular domain of HER3 is the first RNA aptamer selected against an EGFR family member. It was found that high-affinity binding of A30 inhibits heregulin-dependent activation of the pathway and growth of breast cancer MCF-7 cells (Chen et al., 2003). By using purified extracellular domain of human EGFR, Ellington's group has initially identified a predominant RNA aptamer (J-18) with a K_d of about 7 nM (Li et al., 2010). They found this RNA aptamer is able to specifically bind and deliver gold particles into cancer cells expressing EGFR. To further identify RNA aptamers for potential in vivo usage, they performed similar experiments but used more stable 2'-fluoropyrimidine modified RNA aptamer libraries (Li et al., 2011). In this screen, they identified a RNA aptamer (E07) with even higher binding ability (K_d = 2.4 nM). They found that E07 is able to compete with epidermal growth factor (EGF) for binding to EGFR or EGFRvIII, a mutant form of EGFR found on breast and lung cancer, as well as Glioblastoma Multiforme (GBM). It is also worth mentioning that GBM is one of the most common and aggressive malignant brain tumors in adults, but is largely unresponsive to current therapy. In this study, they have shown this aptamer could be a promising candidate for anti-tumor therapy because it is able to not only block EGFR activation and but also prevent tumor cell proliferation in 3D culture. HER2 is often amplified and over-expressed in human breast cancer and has proven to be a key

therapeutic target for the disease (Kang, 2009). Most recently, by using HER-2 over-expressing cells and counter selection with these cells pre-treated with HER-2 siRNA and HER-2 negative cells, Kang et al have used the whole cell SELEX approach and isolated several high affinity RNA aptamers. Although further functional tests are still needed, these and above mentioned RNA aptamers against EGFR family members could have important therapeutic and diagnostic implications in the future.

3.1.2 RNA Aptamers as delivery tools for targeted therapy

Recent isolation of RNA aptamers against cell surface markers of various human diseases has led to the application of these RNA aptamers for targeted delivery of RNA therapeutics, especially those based on RNA interferences (RNAi): small interference RNA (siRNA), short-hairpin RNA (shRNA) or microRNA(miRNA) (Castanotto and Rossi, 2009). RNAi was initially discovered in the nematode worm Caenorhabditis elegans and later confirmed in mammalian cells by using synthetic double stranded small RNA around 20-30 nt in length (Zamore et al., 2000). Once inside the cytoplasm of the cell, siRNA or shRNA is recognized by a protein complex called RNA-induced silencing complex (RISC), which led to its activation and eventual cleavage of the targeted mRNA, thus preventing its protein production (Matzke and Birchler, 2005; Tolia and Joshua-Tor, 2007). With RNAi technology, it has become possible to specifically target virtually all desired cellular proteins of any given pathway in spite of its functions or cellular localization. However, despite these promises, siRNAs, shRNAs or miRNAs first need to be delivered into the cytosol of the targeted cells for them to function, which has become one of the major focuses of RNAi-based therapies. In this regard, RNA

aptamers have provided a unique tool not only for the recognition of specific target cell but also for the delivery of siRNAs and shRNAs into these cells.

3.1.2.1 Prostate Specific Membrane Antigen

Prostate Specific Membrane Antigen (PSMA) is a prostate cancer marker with increased expression on the surface of prostate cancer cells and the tumor vascular endothelium, but not normal prostate epithelia (McNamara et al., 2006). The RNA aptamers (A9 and A10) against PSMA have been isolated and subsequently used for intracellular delivery of therapeutics (siRNAs) in multiple studies (Lupold et al., 2002). Chu et al have non-covalently linked biotinylated A9 aptamer with siRNA against lamin A/C or GAPDH through streptavidin (Chu et al., 2006). They observed successful knockdown of these target genes by the Aptamer:siRNA conjugates in PSMA expressing but not PSMA nonexpressing prostate cancer cells, indicating specific targeting of these particles. McNamara et al conjugated RNA aptamer A10 to siRNAs against cancer survival genes polio-like kinase 1 (*PLK1*) and *BCL2* and tested them on prostate cancer cell growth both in vitro and in vivo (McNamara et al., 2006). They found that these A10-siRNA chimeras were successfully internalized and processed by Dicer, which led to silencing of the expression of these survival genes and subsequent cell death in vitro (McNamara et al., 2006). They went on to further inject the A10-Plk1 siRNA and control mutantA10-PlK1 siRNA chimeras into prostate tumors in an in vivo mouse prostate cancer xenograph model. It was found that A10-Plk1 siRNAs, but not mutantA10-PlK1 siRNAs, are able to significantly inhibit the prostate tumor growth and mediate tumor regression. In subsequent work, Dassie et al have incorporated modifications, added 3' overhangs, truncated the aptamer sequences to optimize and enhance chimeras' thermodynamic profile and circulating half life for systemic

administration, and observed more pronounced regression of PSMA-expressing tumors in vivo (Dassie et al., 2009).

Most recently, this PSMA aptamer has also been reported to deliver siRNAs to induce tumor immunity in one study, and to enhance ionized radiation sensitivity in another for prostate cancer therapy (Ni et al., 2011; Pastor et al., 2010). In the first study, Pastor et al used A10 aptamer to deliver siRNA against Upf2 and Smg1, key components of the RNA surveillance pathway called non-sense mRNA decay (NMD) pathway (Pastor et al., 2010). NMD pathway normally functions to prevent mRNAs expression from premature termination. Disruption of this process can lead to generation of antigens recognized by the immune system as foreign and their subsequent immune-mediated rejection. They found that A10 conjugated Upf2 or Smg1 siRNA can be specifically targeted to tumor cells and inhibit their growth in both cell culture and in vivo xenograft studies. Significantly, the ability of A10-Smg1 to inhibit tumor growth was superior to that of vaccination with granulocyte–macrophage colony-stimulating factor (GM-CSF)-expressing irradiated tumor cells, and could be further enhanced by co-stimulation. In the second study, Ni et al used A10 aptamer-shRNA chimera to target DNA-activated protein kinase (DNA-PK) to increase the radiosensitivity of prostate cancer cells expressing PSMA (Ni et al., 2011). It is well known that ionizing radiation causes damage to cell DNA such as inducing breaks in the double stranded DNA or changes in the DNA sequence. Although ionizing radiation has proven to be a powerful tool in cancer therapy, it is highly nonspecific and also damages normal cells. It is also known that cells have developed ways to correct these DNA damage insults to its genome by means such as double-strand break repair and excision repair. Therefore, if the cells are incapable of repairing the damage induced by radiation, they will likely lose their ability to withstand the radiation and will require less radiation for their treatments. Ni

et al carried out a siRNA library high throughput screen to identify the key genes responsible for the cell's repair mechanisms in PSMA expressing prostate cancer cells and found DNA-PK as one of such target. They found that A10-3 aptamer-DNA-PK shRNA can specifically reduce DNAPK in prostate cancer cells in vitro, in vivo in xenograft models, and even in human prostate tissues. It has also been shown that intravenous administration of A10-3-DNAPK shRNA chimeras greatly sensitized the PSMA positive prostate tumor to ionized radiation. Importantly, it allows the levels of radiation necessary to treat the cancer to be low enough to significantly decrease the damage to surrounding tissues such as the bladder and rectum.

3.1.2.2 CD4 aptamer and pRNA nanodelivery system

Although RNA-based therapy has become a promising avenue for the treatment of many human diseases, a major challenge that remains is the systemic and intracellular delivery of these moieties (siRNA, ribozyme etc) to the desired target cells. RNA aptamers have provided a unique capability for specific cellular targeting but their sub-nano size, even after conjugation to these RNA therapeutics, subjects them to body clearance. In this regard, the pRNA nanoparticle delivery system pioneered by Dr. Guo provided an ideal method for nanoscale delivery suitable for in vivo delivery (Guo, 2010). Since pRNA nanoparticles are composed of all RNA, it is the most natural choice for the delivery of RNA therapeutics because using an all RNA delivery system will also allow all the advantages of RNAs as therapeutic agents to be retained.

pRNA monomer is a 117-nt long RNA molecule about 11nm in size and constitutes one of the six subunits of DNA packaging motor of bacteriophage phi29. pRNA has two functional domains that can fold independently: DNA translocation domain and prohead binding domain. The DNA translocation domain is composed of a 3'/5' double helix loop while left- and right-

hand loops form the prohead binding domain. By replacing the 3'/5' double helix loop of the pRNA sequence with siRNA against a pro-survival gene called survivin, Dr. Guo and colleagues have first demonstrated that it can fold properly to knockdown the expression of target genes and inhibit tumor growth both in vitro and in vivo. One important feature of this pRNA system is its ability to form pRNA dimers and multimers through right-hand loop (A) and left hand loop (B) interactions. Taking advantage of that, Dr. Guo and colleagues generated pRNA dimers with one pRNA(a'-B) carrying RNA aptamer against CD4 receptor and the other pRNA(A-b') conjugated with siRNA against survivin. They found this dimer is able to specifically target CD4 positive lymphocytes to silence the target gene expression and reduce cell viability (Guo et al., 2005).

Another key obstacle for RNA-based therapy is that RNA is susceptible to quick degradation in the blood stream during in vivo delivery. To overcome that, Dr. Guo's laboratory has developed a RNAse-resistant form of pRNA by 2'-deoxy-2'-fluoro (2'-F) modification at the ribose rings of C and U (Liu et al., 2011). They found that 2'-F modified pRNAs are highly stable and can fold into its 3-D structure properly. Most importantly, they have shown that this 2'-F modification of pRNA does not alter its functions and is fully biologically active. This RNAse-resistant phi29 pRNA has also recently been tested for pharmacological characteristics in systemic delivery (Abdelmawla et al., 2011). It was found that 2'-F-modified pRNA nanopaticles are very stable chemically and metabolically in vivo in mice. Most recently, Dr. Guo's laboratory has discovered that the three way junction (3WJ) of pRNA that connects its left hand loop, right hand loop and helix domain can form a complex that is most stable structure found among 25 3WJ motifs obtained from different biological systems (Shu et al., 2011). They have shown that each arm of the 3WJ-pRNA can carry the above mentioned CD4 receptor

binding RNA aptamer, siRNA or ribozyme and bring them into target cells both in vitro and in vivo. Importantly, they have further gone on to show that the 2'-F RNAse resistant form of 3WJ-pRNA also retains its folding and can carry these incorporated functional moities to target cells both in vitro and in vivo.

3.1.2.3 gp120

John Rossi's group used a RNA aptamer against gp120 for targeted delivery of siRNA in their fight against Human Immunodeficiency Virus (HIV) infections (Zhou et al., 2008; Zhou and Rossi, 2011; Zhou et al., 2011; Zhou et al., 2009). When a person is exposed to and infected with HIV-1, the virus enters the individual's body and targets cells that express the CD4 receptor. The HIV-1 virus uses its surface protein called glycoprotein gp120 to recognize the CD4 cell receptor and initiate the membrane fusion and subsequent delivery of viral RNA and enzymes. Once infected, these cells will then also express gp120 on their cell surface. Zhou et al generated gp120 aptamer chimeras with siRNA targeting the HIV-1 tat/rev region. They found the chimera can be specifically internalized into cells expression gp120 to silence the target gene expression. Importantly, these gp120 aptamer-siRNA chimeras exhibited potent and lasting effect on inhibiting HIV replication in T cells without triggering interferon response. Rossi's group recently used the same pRNA system developed by Dr. Guo, as described above, to generate dual functional RNA nanoparticles and also achieved both cell type specific delivery and targeted inhibition (Zhou et al., 2011). In this study, they used pRNA(a'-B) to form a chimera with RNA aptamer that recognize gp120 and linked siRNA against HIV-1 tat/rev to the complementary pRNA(A-b'). They found the pRNA(a'-B)-aptamer chimera could specifically bind to HIV infected cells and that pRNA(A-b')-siRNA chimera could be processed by Dicer as

expected. They further reported that incubating the pRNA(a'-B)-aptamer/pRNA(A-b')-siRNA dimers with HIV infected cells led to successful binding by the aptamer and delivery of the siRNA. They have also applied 2'-F modified pyrimidines in the sense strand of pRNA-siRNA chimera and found the chimera to be more stable in the serum. Importantly, the modified chimera can still be functionally processed by Dicer to specifically silence the target gene expression, thus paving the way for future in vivo systemic delivery studies.

3.2 Diagnostic Applications of RNA Aptamers

Use of RNA aptamers has already been successful in treating AMD and has shown promise in treating prostate cancer, HIV infection and other diseases, while further research will undoubtedly be done to target more cell components for disease treatment. Importantly, in addition to these therapeutic applications, RNA aptamers have also started to play increasingly important roles not only in environmental and food analysis, but also in human disease diagnosis (Dua et al., 2011; Jayasena, 1999; Soontornworajit and Wang, 2011; Tombelli et al., 2007). In this section, we will focus on the role of RNA aptamers in disease diagnosis through imaging, disease cell detection and novel biomarker discovery.

3.2.1 RNA aptamer as an imaging tool

Antibodies have long been the focus of such diagnostic studies because of their ability to target certain cell markers. With the development of RNA nanotechnology and the SELEX procedure, RNA aptamers have become a more attractive option when compared to their antibody counterparts. RNA aptamers used for imaging have the same advantages of those used for therapy: low immunogenicity, smaller size, and relatively short time to develop the desired

aptamer(Pieve et al., 2009). Imaging via selective delivery of radionucleotides to tumors has been investigated for many years. When attaching these radioactive substances to aptamers and antibodies, the residual radioactive aptamers in the bloodstream are quickly cleared and excreted, owing to the rapid uptake of Aptamers by tumors. Not only does this allow for superior tumor imaging, but it also decreases the toxicity to normal tissues that was often seen with radioactively labeled antibodies due to their slow clearance from the body (Pieve et al., 2009).

One such example of imaging applications is the RNA aptamer for tenascin-C, an extracellular matrix protein upregulated in a number of tumors such as breast, lung, colon, prostate, glioblastoma, and lymphoma (Dua et al., 2011; Levy-Nissenbaum et al., 2008). An anti-TN-C aptamer, TTA1, was discovered, purified, and then modified to increase stability in serum and to allow for subsequent radionucleotide conjugation. Researchers have used radionucleotide ^{99m}Tc to image the cells that are able to bind to and internalize the TTA1 aptamer. They found TTA1-^{99m}Tc exhibited high "signal to noise ratio" as it was quickly uptaken by the tumor cell and eliminated from the bloodstream. Importantly, the tumor retention time of TTA1-^{99m}Tc was sufficiently long that the tumor image was much clearer compared to background when imaged using single-photon emission-computed tomography (Levy-Nissenbaum et al., 2008). Another example of RNA aptamers that have potential for tumor imaging in humans is the above mentioned A10 aptamer against prostate cancer marker PMSA. A10 aptamer has been tested for tumor imaging after conjugation with quantum dots, as well as thermally cross-linked supraparamagnetic iron oxide nanoparticle (TCL-SPION) (Dua et al., 2011). TCL-SPION nanoparticle is often used as a contrast agent in MRI imaging, and has low systemic toxicity. In this case, A10 aptamer allows TCL-SPION to bind specifically to prostate

cancer cells expressing PMSA thus enables the imaging capabilities of TCL-SPION to be localized to the prostate tumor.

3.2.2 RNA Aptamers in disease diagnosis

In addition to their role as imaging tools, RNA aptamers can also aid in clinical diagnosis of diseases due to their high affinity to bind specific cell markers. Their small size, stable structure and ease of synthesis also add to their attraction to detect human diseases, even before the symptoms become apparent. Importantly, the easy conjugation and labeling features of RNA aptamers also allow them to be combined with other advanced technologies such as microfluidic cell separation, endogenous nucleic acid analysis, nanoparticle based sensing or flow cytometry to maximize their diagnostic functions (Jayasena, 1999; Soontornworajit and Wang, 2011).

Recently, RNA aptamers have become an attractive tool in detecting diseased cells on a histological section and, most importantly, the presence of very low amounts of circulating disease cells in the bloodstream. One such example is to use the above mentioned RNA aptamer against EGFR to determine the presence or extent of GBM, a deadly disease that is hard to detect. To achieve that, Wan et al first immobilized the aptamer on a chemically modified glass surface and exposed it to the cells in question, either from serum or the tumor margin (Wan et al., 2010). The bound cells were then collected and imaged on a neuro-optical microfluidic platform to quantify the disease cells and determine the extent of disease. Through these procedures, they were able to detect primary human GBM cells expressing high levels of EGFR with high sensitivity and specificity. Therefore, this approach could lead to earlier diagnosis of this highly malignant tumor and monitoring of residue disease after the treatment by detecting circulating tumor cells in the serum. In the case of a tumor resection, this would also allow the surgeon to know whether surgical resection margins of the tumor are free from diseased cells.

RNA aptamers have also shown potential for use in conjunction with flow cytometry to detect diseased cells. Li et al have recently tested RNA aptamers against Human EGFR Related 3 (HER3), Tn-C, PSMA, and EGFR for detection of varieties of human cancer cells (Barfod et al., 2009). They optimized the assay and received strong signals by fluorescent labeling of biotinylated RNA aptamers with streptavidin-phycoerythrin (SA-PE) for flow cytometry (Barfod et al., 2009). Although the ability of these RNA aptamers to detect different cancer cell varies and optimization will likely be required for each application, they nevertheless show promise for use of RNA aptamers with flow cytometry to detect a wide variety of human cancer cells. However, more work may still need to be done to improve the current technology, especially because relative to normal cells in a blood sample, there are very few diseased cells, making detection of these cells more difficult. To overcome that, it may be necessary to perform additional steps of *ex vivo* amplification of cells before passing them through the flow cytometer. In addition, further work is still needed to ensure single cells pass through the flow cytometer, as the cell clusters often naturally occur and skew the results (Brown and Wittwer, 2000; Ringquist and Parma, 1998).

Diagnostically, aptamers can also be utilized in a similar manner that antibodies have been used in a two-site binding assay, the most commonly used diagnostic format today. Using this approach, Drolet et al were able to detect serum vascular endothelial growth factor (VEGF) protein, which plays an important role in angiogenesis and has been used as biomarker for breast cancer, lung cancer and colorectal cancer (Drolet et al., 1996). In this assay, RNA aptamers targeting VEGF are synthesized and labeled with fluorescein. Alkaline phosphatase conjugated Fab antibody fragments directed against fluorescein were then used to detect fluorescein labeled RNA aptamers. Li et al recently used a different approach and were able to detect VEGF at a

biologically relevant concentration of 1 pM (Li et al., 2007). They used immobilized RNA aptamer arrays to enrich serum VEGF, followed by signal amplification using horseradish peroxidase (HRP) conjugated antibodies against VEGF and measurements by surface plasmon resonance imaging (SPRI). Although there is still significant work needed to be done before these and above mentioned approaches can be used clinically, it is clear that RNA aptamer technology has the potential to make early detection of disease possible, and with further development, become indispensable to the medical field.

3.2.3 RNA Aptamers in Biomarker Discovery

Identifying and characterizing cell markers for diagnosis and treatment purposes have historically been difficult. As mentioned above, there has been a number of RNA aptamers recently isolated that can be used as biomarkers to differentiate between different cell types or distinguish disease cells from normal cells. In addition, RNA aptamers also have a unique advantage in novel biomarker discovery because RNA can be selected through the SELEX procedure without prior knowledge of these cell markers (Dua et al., 2011; Zhang et al., 2010). Current studies combining 2D gel electrophoresis and mass spectrometry have made it relatively easy to identify soluble biomarkers. However, it still requires a significant amount of these biomarkers, often expressed at low levels to be isolated for identification. In addition, the amphipathic nature of cell membrane proteins has made identification of cell surface markers using this approach insufficient, because the extraction of these cell surface proteins requires detergent that tends to diminish the signal from mass spectrometry (Dua et al., 2011). By immobilizing RNA aptamers, several methods have recently been developed that can be used to

enrich both soluble and membrane biomarkers for subsequent mass spectrometry analysis (Bagalkot et al.; Windbichler and Schroeder, 2006).

Remarkably, Mi et al most recently developed a unique approach to isolate RNA aptamers in vivo against tumor cells in living mice and subsequently used selected RNA aptamers to identify the biomarker for hepatic colon cancer metastases. In this study, they intravenously injected 2'fluro-pyrimidine modified RNA aptamer library into an animal model of intrahepatic colorectal cancer metastases, where mice were implanted with hepatic tumor. Liver tumors were then harvested for RNA molecules extraction and amplification. The resulting pool of RNA was then reinjected and repeated the above process for 14 rounds. They found that RNA aptamer 14-16 was able to specifically stain intrahepatic CT26 tumors both in vitro and in vivo. To further identify the tumor specific protein that RNA aptamer 14-16 interacts with, they further immobilized biotinylated RNA apatmers on streptavidin magnetic beads and then incubated with tumor tissue extracts. After standard washing and gel electorphoresis steps, they are able to use peptide-mass fingerprinting and MS/MS peptide fragment ion-matching to determine that RNA 14-16 binds to Ddx5, a p68 RNA helixase that has previously been reported to overexpressed in colorectal tumors. With these developments, it becomes quite clear that RNA aptamers have great potential in biomarker discovery and, with further developments and refinement, are poised to become a more mainstream approach and lead to more cell-marker targets for disease diagnosis and treatment.

Conclusions and Future Perspective

Although antibody have proven to be a powerful tool not only in diagnosis but also in disease therapeutics, the high affinity and specificity of RNA aptamers rival antibodies and make

them a promising tool in diagnostic and therapeutic application, as evidenced by ample examples given above. With their many advantages over antibodies, such as the small size, high stability, ease and consistency of in vitro synthesis, multi-conjugation capability with other moieties (fluorescein, RNA nanoparticles, etc.) and non-immunogenic nature, RNA aptamers will no doubt find more applications that can be used in conjunction with or complement to antibodies in this areas. We should expect more RNA aptamers be isolated in the near future against an ever increasing repertoire of targets, using these different SELEX approaches with increased speed and efficiency. With the first RNA therapeutics approved by FDA, we should expect more of them to follow. These RNA aptamers could be used either to block key cellular pathways or as a delivery tools for other RNA nanotechnology based therapeutics. With increasing interest and further improvement of RNA aptamers and RNA nanotechnology, we should also expect wider diagnostic applications using RNA aptamers in imaging, disease detection and biomarker discovery in the years to come.

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Figure Legend

Figure 1. An overview of the SELEX procedures. A) SELEX: A RNA pool undergoes negative and positive selection (gray arrows), followed by reverse transcription, PCR amplification, and T7 transcription (black arrows). B) Cell-SELEX, C) Crossover-SELEX, and D) Tissue SELEX instead use B) whole cells, C) whole cells plus protein markers, or C) tissue sections or live animals for the selection step(s), followed by the steps outlined by the black arrows as in A). All of the above procedures are usually repeated for 5-15 cycles.

Table I. A list of RNA aptamer sequences and their targets.

Aptamer target	Aptamer sequence (5' → 3')	Disease Target/ Applications	Ref.
4,4'-methylenedianiline (MDA)	CUGCGAUCAGGGGUAAAUUUCCGCGCAGGC UCCACGCCGC	MDA is an aromatic carcinogen	(Brockstedt et al., 2004)
Acetylcholine receptor (AChR)	GCUAGUAGCCUCAGCAGCAUAGUUUCGCCGC UAUGCAGUA	Neuromuscular disorder	(Ulrich et al., 1998)
African trypanosomes	AUCGCUACUGCGCCGGUUGCGGCUUGCGGUU GCAACGCCA	Chagas' disease	(Ulrich et al., 2002)
AMPA receptor GluR2Qflip	GGGCGAAUUCAACUGCCAUCUAGGCAGUAA CCAGGAGUUAGUAGGACAAGUUUCGUCC	Cerebral ischemia, Atrophic Lateral Sclerosis (ALS)	(Huang et al., 2007)
Beta Secretace (S10)	GGGAUAGGAUCCACAUCUACGUAUUAGUAC ACGUCGGCCACCUACGCGAAGUG GAAGCCUCAUUUGUUCACUGCAGACUUGAC GAAGCUU	Alzheimer's Disease	(Rentmeister et al., 2006)
Beta Secretase (TH14)	GGGAUAGGAUCCACAUCUACGUAUUACGCA ACGCCGGGCCACUACGCGAAUGGC AAGCCCGUCGACUUCACUGCAGACUUGACGA AGCUU	Alzheimer's disease	(Rentmeister et al., 2006)
CD4	CUCAGACAGAGCAGAAACGACAGUUCAAGCC GAA	HIV	(Zhou et al., 2008)
CTLA-4	GGGAGAGAGGAAGAGGGAUGGGCCGACGUG CCGCAACUUCAACCCUGCACAACCAAUCCGC CCAUAACCCAGAGGUCGAUAGUACUGGAUCC CCCC	Cancer	(Santulli-Marotto et al., 2003)
EGFR (E07)	UGCCGCUAUAAUGCACGGAUUUAAUCGCCG UAGAAAAGCAUGUCAAAGCCG	Cancer	(Li et al., 2011)
EGFR (J18)	GGCGCUCCGACCUUAGUCUCUGCAAGAUAAA CCGUGCUAUUGACCACCCUCA ACACACUUAUUUAAUGUAUUGAACGGACCU ACGAACCGUGUAGCACAGCAGA	Cancer	(Li et al., 2009; Li et al., 2010)
EGFRvIII (E17)	ACCAAAAUCAACGCAAAGAGCGCGCCUGCAC GUCACCUCA	Cancer	(Liu et al., 2009)
Erythrocyte membrane protein 1 (PfEMP1)	GGGAAUUCGACCUCGGUACCAACAAUACGAC UACACCAUCAAAAGUAUUAUCUUGCAUCGA AGGUUGGCGUAGCAAGCUCUGCAGUCG	Malaria	(Barfod et al., 2009)
gp120	GGGAGACAAGACUAGACGGUGAAUGUGGGC CACGCCCGAUUUUACGCUUUUACCCGCACGC GAUUGGUUUGUUUCCC	HIV Infection	(Khaled et al., 2005)
HER3	GGGAAUUCCGCGUGUGCCAGCGAAAGUUGC GUAUGGGUCACAUCGCAGGCACAUGUCAUC UGGGCGGUCCGUUCGGGAUCCUC	Breast cancer	(Li et al., 2009)
Human keratinocyte growth factor	CCCAGGACGAUGCGGUGGUCUCCCAAUUCUA AACUUUCUCCAUCGUAUCUGGG	Cancer,	(Pagratis et al., 1997)
L-selectin	UAACAACAAUCAAGGCGGGUUCACCGCCCCA GUAUGAGUA	Inflammation, Post-ischemic	(O'Connell et al.,

		processes	1996)
Neruotensin-1 (NTS-1)	ACAGATACGGAACTACAGAGGTCAATTACGG TGGCCACGC	Neurologic Diseases	(Daniels et al., 2002)
NF-κB	CAUACUUGAAACUGUAAGGUUGGCGUAUG	Inflammatory, Cancer	(Huang et al., 2003)
Phosphatidylcholine:cholesterol liposomes	GGGAUCUACACGUGACUGACUUACGAGACU GUCUCGCCAAUUCCAGUGGGCCUGCGGAUCC U	Membrane permeability	(Khvorova et al., 1999)
PSMA (A10)	GGGAGGACGAUGCGGAUCAGCCAUGUUUAC GUCACUCCUUGUCAAUCCUCAUCGGCAGACU CGCCCGA	Prostate cancer	(McNamara et al., 2006)
Raf-1	GGGAGAUCGAAUAAACGCUCAAUUUGCCUC GACGGUCUGCGAAUAGAACGCGAACCGUGA UUAGUGUACAAGGAUUCGGUUUUCGACAUG AGGCCCCUGCAGGGCG	Cancer	(Kimoto et al., 1998)
RET receptor tyrosine kinase	GCGCGGGAATAGTATGGAAGGATACGTATAC CGTGCAATCCAGGGCAACG	Multiple endocrine neoplasia	(Cerchia et al., 2005)
TCF-1	GGGGAGCUCGGUACCGGUGCGAUCCCCUGUU UACAUUGCAUGCUAGGACGACGCGCCCGAGC GGGUACCGAUUGUGUCGUCGGAAGCUUUGC AGAGGAUC	Colon cancer	(Choi et al., 2006)
Tenascin-C (AptamerTTA1)	GGGAGGACGCGUCGCCGUAAUGGAUGUUUU GCUCCCUG	Glioblastoma and Breast cancer	(Hicke et al., 2006)
TGF-β type III receptor	GGGCCAGGCAGCGAGAGAUAAGCAGAAGAA GUAUGUGACCAUGCUCCAGAGAGCAACUUC ACAUGCGUAGCCAAACCGACCACACGCGUCC GAGA	Ovarian cancer	(Ohuchi et al., 2006)
Tumor necrosis factor superfamily member 4-1BB	GGGAAGAGAGGAAGAGGGAUGGGCGACCGA ACGUGCCCUUCAAAGCCGUUCACUAACCAGU GGCAUAACCCAGAGGUCGAUAGUACUGGUC CCCCC	Mastocytoma	(McNamara et al., 2008)
Tumor necrosis factor superfamily member OX40	GGGAGGACGATGCGGCAGUCUGCAUCGUAG GAAUCGCCACCGUAUACUUUCCCACCAGACG ACUCGCUGAGGAUCCGAGA	Cancer	(Dollins et al., 2008)
VEGF	CGGAAUCAGUGAAUGCUUAUACAUCCG	Age related macular degeneration	(Lee et al., 2005)
Wilms tumor protein (WT1)	GAUAUGGUGACCACCCCGGC	Wilms tumor	(Bardeesy and Pelletier, 1998)
$\alpha_v\beta_3$ integrin	GGGAGACAAGAAUAAACGCUCAAUUCAACG CUGUGAAGGGCUUAUACGAGCGGAUUACCC UUCGACAGGAGGCUCACAAAAGGC	Anti-cancer, anti-thrombotic, anti-inflammatory	(Mi et al., 2005)
β-catenin	GGACGCGUGGUACCAGGCCGAUCUAUGGAC GCUAUAGGCACACCGGAUACUUUAACGAUU GGCUAAGCUUCCGCGGGGAUC	Colon cancer	(Lee et al., 2006)

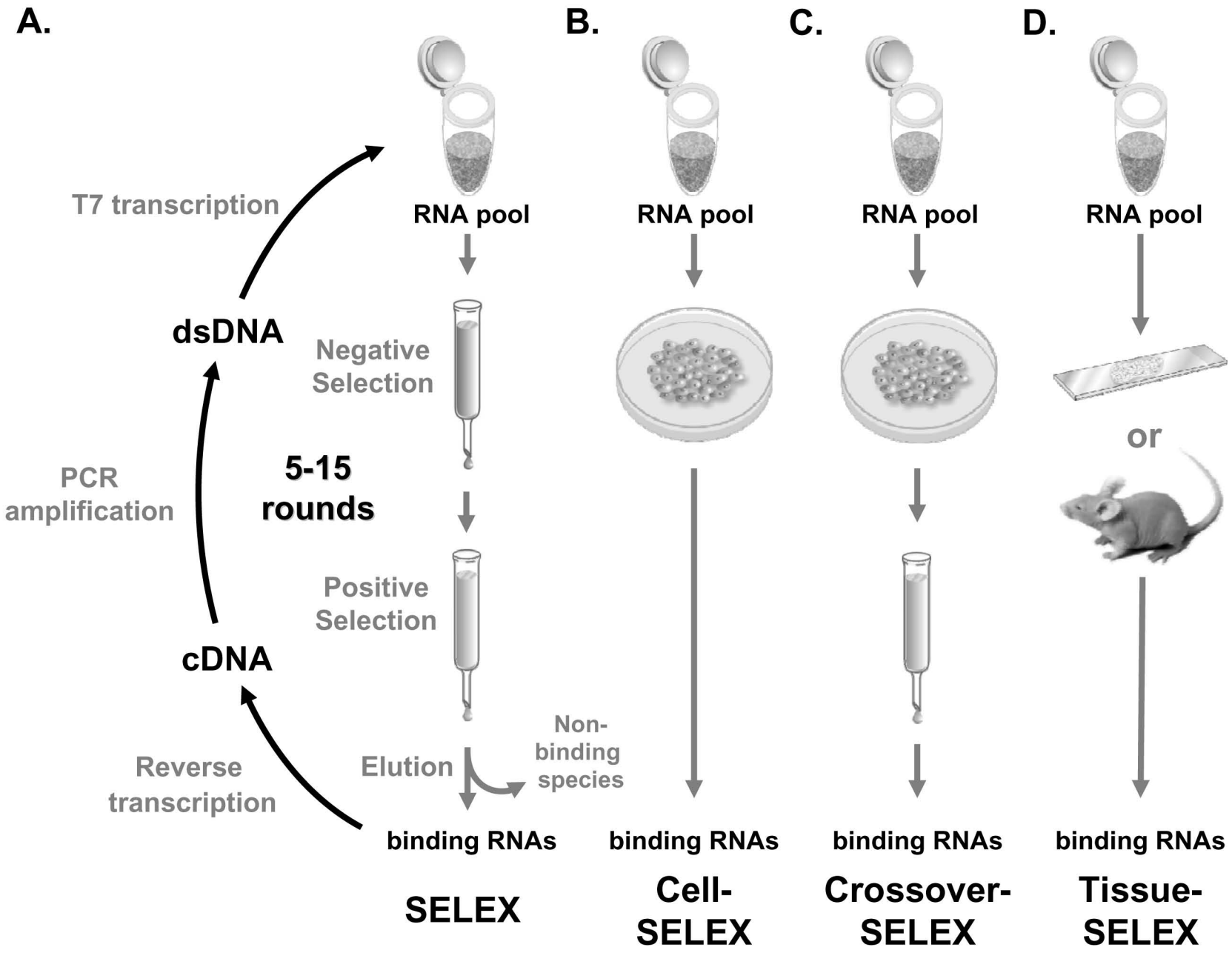
A.
B.
C.
D.
RNA pool
RNA pool
RNA pool
RNA pool
T7 transcription
dsDNA
Negative Selection
5-15 rounds
PCR amplification
Positive Selection
cDNA
Elution
Non-binding species
Reverse transcription
or
binding RNAs
binding RNAs
binding RNAs
binding RNAs
SELEX
Cell-SELEX
Crossover-SELEX
Tissue-SELEX

Figure 1