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Review Article

RNA aptamers and their therapeutic and diagnostic applications

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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 article summarizes the current advancement in the identification of RNA aptamers and also provides some examples of their therapeutic and diagnostic applications.

Keywords: RNA nanotechnology, RNA aptamer, SELEX, RNA therapy, drug delivery

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 [1, 2]. These RNA molecules were termed as "aptamers," with etymology stemming from the Greek word *aptus*, which means "to fit" [1, 3]. 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 [4-11].

Although this review article 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 [4, 6, 9, 12]. 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 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.

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 [1-3]. 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 [5-7, 9].

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 [1-3, 5, 7, 10]. 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 transcribed 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 [13]. This not only made it possible to complete a SELEX experiment in a matter of days, but also improved the consistency of results.

Cell-SELEX

There are also different variations of the SELEX procedure that can be done; one of which is called Cell-SELEX [5, 7, 10]. 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

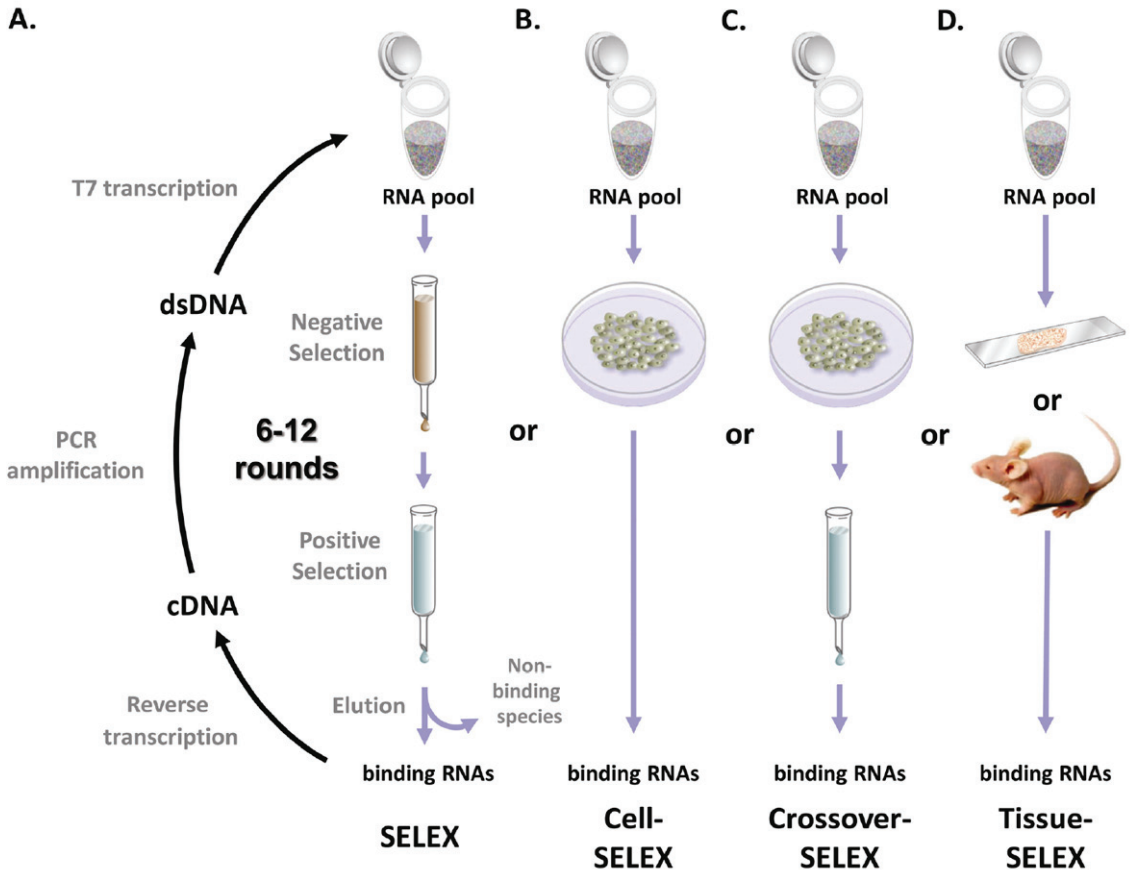

Figure 1. An overview of the SELEX procedures. A. SELEX: A RNA pool undergoes negative and positive selection (gray arrows), followed by reverse transcription, PCR amplification, and T7 transcription (black arrows). B. Cell-SELEX, C) Crossover-SELEX, and D) Tissue SELEX instead use B) whole cells, C) 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 6-12 cycles.

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 decreases the efficiency of the process [5].

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 [5, 7, 10].

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 [5]. This process is accomplished by exposing diseased tissue section slides to the pool of aptamers. The healthy tis-

sues 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 [14].

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 1**) [4, 6, 7, 9]. 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.

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 [4, 6, 7, 10, 12]. 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.

RNA aptamers as therapeutic agents

With the advancement of RNA nanotechnology and development of RNA aptamers suitable for systematic delivery, RNA aptamers have become attractive thereaputic agents against many targets, especially those in the ocular compartments, blood stream and cell surface proteins [4, 6]. 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) [15]. 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.

Vascular endothelial growth factor: Vascular Endothelial Growth Factor (VEGF) is a protein that plays essential roles in both physiologic and pathologic angiogenesis [16-19]. 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) [15]. Taking advantage of that, researchers have carried out studies to isolate the RNA aptamer that specifically targets the $VEGF_{165}$ isoform [20-22]. 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 [15]. Preclinical 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

Table 1. A list of RNA aptamer sequences and their targets

Aptamer target	Aptamer sequence (5' → 3')	Disease Target/ Applications	Ref.
4,4'-methylenedianiline (MDA)	CUGCGAUCAGGGGUAAAUUUCCGCGCAGGCUCCACGCCGC	MDA is an aromatic carcinogen	[61]
Acetylcholine receptor (AChR)	GCUAGUAGCCUCAGCAGCAUAGUUUCGCCGCUAUGCAGUA	Neuromuscular disorder	[62]
African trypanosomes	AUCGCUACUGCGCCGGUUGCGGCUUGCGGUUGCAACGCCA	Chagas' disease	[63]
AMPA receptor GluR2Qflip	GGGCGAAUUCAACUGCCAUCUAGGCAGUAACCAGGAGUUAGUAGGACAAGUUUCGUCC	Cerebral ischemia, Atrophic Lateral Sclerosis (ALS)	[64]
Beta Secretace (S10)	GGGAUAGGAUCCACAUCUACGUAUUAGUACACGUCGGCCACCUACGCGAAGUG GAAGCCUCAUUUGUUCACUGCAGACUUGACGAAGCUU	Alzheimer's Disease	[65]
Beta Secretase (TH14)	GGGAUAGGAUCCACAUCUACGUAUUACGCAACGCCGGGCCACUACGCGAAUGGC AAGCCCGUCGACUUCACUGCAGACUUGACGAAGCUU	Alzheimer's disease	[65]
CD4	CUCAGACAGAGCAGAAACGACAGUUCAAGCCGAA	HIV	[46]
CTLA-4	GGGAGAGAGGAAGAGGGAUGGGCCGACGUGCCGCAACUUCAACCCUGCACAACCAAUCCGCCCAUAACCCAGAGGUCGAUAGUACUGGAUCCCCCC	Cancer	[66]
EGFR (E07)	UGCCGCUAUAAUGCACGGAUUUAAUCGCCGUAGAAAAGCAUGUCAAAGCCG	Cancer	[30]
EGFR (J18)	GGCGCUCCGACCUUAGUCUCUGCAAGAUAAACCGUGCUAUUGACCACCCUCA ACACACUUAUUUAAUGUAUUGAACGGACCUACGAACCGUGUAGCACAGCAGA	Cancer	[29, 67]
EGFRvIII (E17)	ACCAAAAUCAACGCAAAGAGCGCGCCUGCACGUCACCUCA	Cancer	[68]
Erythrocyte membrane protein 1 (PfEMP1)	GGGAAUUCGACCUCGGUACCAACAAUACGACUACACCAUCAAAAGUAUUAUCUUGCAUCGAAGGUUGGCGUAGCAAGCUCUGCAGUCG	Malaria	[53]
gp120	GGGAGACAAGACUAGACGGUGAAUGUGGGCCACGCCCGAUUUUACGCUUUUACCCGCACGCGAUUGGUUUGUUUCCC	HIV Infection	[69]
HER3	GGGAAUUCCGCGUGUGCCAGCGAAAGUUGCGUAUGGGUCACAUCGCAGGCACAUGUCAUCUGGGCGGUCCGUUCGGGAUCCUC	Breast cancer	[67]
Human keratinocyte growth factor	CCCAGGACGAUGCGGUGGUCUCCCAAUUCUAAACUUUCUCCAUCGUAUCUGGG	Cancer,	[70]
L-selectin	UAACAACAAUCAAGGCGGGUUCACCGCCCCAGUAUGAGUA	Inflammation, Post-ischemic processes	[71]
Neruotensin-1 (NTS-1)	ACAGATACGGAACTACAGAGGTCAATTACGGTGGCCACGC	Neurologic Diseases	[72]
NF-κB	CAUACUUGAAACUGUAAGGUUGGCGUAUG	Inflammatory, Cancer	[73]
Phosphatidylcholine: cholesterol liposomes	GGGAUCUACACGUGACUGACUUACGAGACUGUCUCGCCAAUUCCAGUGGGCCUGCGGAUCCU	Membrane permeability	[74]
PSMA (A10)	GGGAGGACGAUGCGGAUCAGCCAUGUUUACGUCACUCCUUGUCAAUCCUCAUCGGCAGACUCGCCCGA	Prostate cancer	[36]
Raf-1	GGGAGAUCGAAUAAACGCUCAAUUUGCCUCGACGGUCUGCGAAUAGAACGCGAACCGUGAUUAGUGUACAAGGAUUCGGUUUUCGACAUGAGGCCCCUGCAGGGCG	Cancer	[75]
RET receptor tyrosine kinase	GCGCGGGAATAGTATGGAAGGATACGTATACCGTGCAATCCAGGGCAACG	Multiple endocrine neoplasia	[76]
TCF-1	GGGGAGCUCGGUACCGGUGCGAUCCCCUGUUUACAUUGCAUGCUAGGACGACGCGCCCGAGCGGGUACCGAUUGUGUCGUCGGAAGCUUUGCAGAGGAUC	Colon cancer	[77]
Tenascin-C (AptamerTTA1)	GGGAGGACGCGUCGCCGUAAUGGAUGUUUUGCUCCCUG	Glioblastoma and Breast cancer	[78]
TGF-β type III receptor	GGGCCAGGCAGCGAGAGAUAAGCAGAAGAAGUAUGUGACCAUGCUCCAGAGAGCAACUUCACAUGCGUAGCCAAACCGACCACACGCGUCCGAGA	Ovarian cancer	[79]
Tumor necrosis factor superfamily member 4-1BB	GGGAAGAGAGGAAGAGGGAUGGGCGACCGAACGUGCCCUUCAAAGCCGUUCACUAACCAGUGGCAUAACCCAGAGGUCGAUAGUACUGGUCCCCCC	Mastocytoma	[80]
Tumor necrosis factor superfamily member OX40	GGGAGGACGATGCGGCAGUCUGCAUCGUAGGAAUCGCCACCGUAUACUUUCCCACCAGACGACUCGCUGAGGAUCCGAGA	Cancer	[81]
VEGF	CGGAAUCAGUGAAUGCUUAUACAUCCG	Age related macular degeneration	[82]
Wilms tumor protein (WT1)	GAUAUGGUGACCACCCCGGC	Wilms tumor	[83]
$\alpha_v\beta_3$ integrin	GGGAGACAAGAAUAAACGCUCAAUUCAACGCUGUGAAGGGCUUAUACGAGCGGAUUACCCUUCGACAGGAGGCUCACAAAAGGC	Anti-cancer, anti-thrombotic, anti-inflammatory	[84]
β-catenin	GGACGCGUGGUACCAGGCCGAUCUAUGGACGCUAUAGGCACACCGGAUACUUUAACGAUUGGCUAAGCUUCCGCGGGGAUC	Colon cancer	[85]

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 remain in eye 28 days post single intravitereous injection in monkeys [23]. 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.

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) [24]. 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-a, 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 [25]. 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 [26, 27]. 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 [28]. 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 [29]. 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 [30]. 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 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 [31]. 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.

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) [32]. 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 [33]. 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 [34, 35]. 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 pro-

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

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 [36]. The RNA aptamers (A9 and A10) against PSMA have been isolated and subsequently used for intracellular delivery of therapeutics (siRNAs) in multiple studies [37]. Chu et al have non-covalently linked biotinylated A9 aptamer with siRNA against lamin A/C or GAPDH through streptavidin [38]. 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 [36]. 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 [36]. They went on to further inject the A10-Plk1 siRNA and control mutantA10-PIK1 siRNA chimeras into prostate tumors in an in vivo mouse prostate cancer xenograph model. It was found that A10-Plk1 siRNAs, but not mutantA10-PIK1 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 [39].

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 [40, 41]. 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 [40]. 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 [41]. 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.

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 [12]. 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 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 [42].

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 [43]. 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 [44]. 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 the most stable structure found among 25 3WJ motifs obtained from different biological systems [45]. 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.

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 [46-49]. 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 expressing 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 [49]. 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.

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 [5, 8, 11, 50]. In this section, we will focus on the role of RNA aptamers in disease diagnosis through imaging, disease cell detection and novel biomarker discovery.

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 [51]. 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 [51].

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 [5, 7]. 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 [7]. 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) [5]. 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 enabling the imaging capabilities of TCL-SPION to be localized to the prostate tumor.

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 [8, 11].

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 [52]. 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 [53]. They optimized the assay and received strong signals by fluorescent labeling of biotinylated RNA aptamers with streptavidin-phycoerythrin (SA-PE) for flow cytometry [53]. 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 [54, 55].

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 [56]. 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 [57]. 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.

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 [5, 58]. 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 [5]. 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 [59, 60].

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 the process mention above was repeated 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 be 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 antibodies 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 these areas. We should expect more RNA aptamers to 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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