[Frontiers in Bioscience 10, 1802-1827, May 1, 2005]

APTAMERS: PROSPECTS IN THERAPEUTICS AND BIOMEDICINE

Amy C. Yan [1], Kathryn M. Bell [1], Megan M. Breeden [1], and Andrew D. Ellington [1,2]

[1] Institute for Cellular and Molecular Biology, [2]Department of Chemistry and Biochemistry, University of Texas at Austin, Austin, TX 78712-0159

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1. ABSTRACT

Most biopolymer drugs to date have been proteins. However, the ability to select nucleic acid binding species (aptamers) has led to the development of protein inhibitors and modulators that are small, readily synthesized nucleic acids. The techniques for optimizing, stabilizing, and delivering nucleic acid therapies are just beginning to be developed, but the same engineering flexibility that has so far allowed the generation of multiple, high affinity and specificity binding species appears to also apply to the methods for adapting nucleic acids to clinical applications. We review the selection and characterization of various aptamers and their applications to a variety of disease states, and then focus on the hurdles that must be overcome for the use of aptamers as both exogenously delivered drugs and as gene therapies.

2. INTRODUCTION

Aptamers are nucleic acid binding species generated by iterative rounds of *in vitro* selection, or SELEX (Figure 1; reviewed in (1-3)). Briefly, randomized pools of RNA or ssDNA are incubated with target molecules under carefully chosen selection conditions. Binding species are partitioned away from non-binders, amplified to generate a new pool, and the process is repeated until a desired 'phenotype' is achieved or until

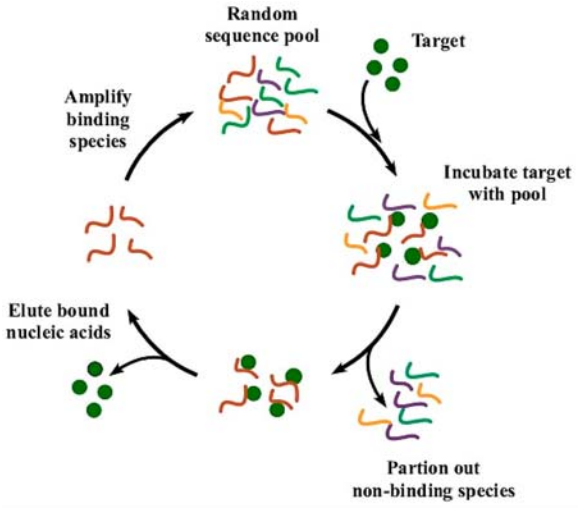

Figure 1. General aptamer selection scheme.

very few sequences remain in the population. Targets for selection have ranged from simple organic molecules to proteins, complexes, and even whole cells (see the Aptamer Database at http://aptamer.icmb.utexas.edu for a comprehensive compilation). Typically, selected aptamers bind very tightly and specifically to their targets. Often, aptamer K_d's for protein targets are in the low nanomolar range, and aptamers have been known to discriminate between very closely related targets. One early example was an aptamer that could bind to theophylline, but not to caffeine, which differs by one methyl group (4). Aptamers can also discriminate between closely related protein targets. Conrad *et al.* selected aptamers that could bind to the beta II isoform of protein kinase C in the nanomolar range, but bound 10-fold less well to the very closely related related beta I isozyme (5). Even better, Tuerk *et al.* selected anti-HIV-reverse transcriptase (RT) aptamers that bound to HIV-1 RT with a K_d of 25 pM, but bound almost four orders magnitude lower to HIV-2 RT, which had 60% sequence identity (6, 7).

By varying key parameters of a selection, aptamers with finely tuned physical and functional properties have been selected, opening the way to an expanded range of applications. Aptamer selections have progressed from targeting isolated proteins and yielding simple proof-of-principle binding species towards targeting cell surfaces or tissues and generating reagents that have more direct applications in manipulating cellular pathways and modulating gene expression. In particular, because of their excellent binding characteristics and general lack of toxicity, aptamers are emerging as promising therapeutic reagents. Aptamers have now been selected against a wide array of biomedically relevant targets (Table 1). Several aptamers have even entered clinical trials. For example, anti-thrombin and anti-nucleolin aptamers are currently in Phase I clinical trials for human coronary bypass surgery and tumorigenesis, respectively (Aptamera, 2004, Nuvelo, Inc., 2004). Anti-VEGF aptamers for treatment of macular degeneration recently became the first aptamers approved by the FDA (as Macugen by Eyetech Pharmaceuticals and Pfizer, Inc. 2004).

In this review, we will first tout some of the features of aptamers that make them especially good therapeutic candidates relative to other biopolymer drugs, such as antibodies, and then examine in greater detail some of the successful selections that have been directed at targets of biomedical relevance.

3. ADVANTAGES OF APTAMERS AS THERAPEUTICS

Aptamers have several key features that make them particularly well suited as drugs. Aptamers are relatively small and can readily access sites on antigens that have previously been difficult to target. As an example, *Trypanosoma brucei*, the causative agent of sleeping sickness, produces a variable surface glycoprotein (VSG) that is difficult for the immune system to generate antibodies against. Nonetheless, aptamers selected against whole trypanosomes *in vitro* were able to access and bind to constant regions located within a flagellar pocket masked by the variable surface (8). While aptamers typically do not bind their targets as tightly as antibodies, this is a good illustration of how *in vitro* selection methods can sometimes generate reagents more readily than *in vivo* methods, such as immunization.

Another advantage of in vitro methods is the ability to craft selections so as to yield aptamers with desired specificity profiles. In the absence of any special effort, the specificities of aptamers can be either wide or narrow. For example, aptamers selected against one Trypanosoma strain were able to target multiple different strains. In order to ensure such breadth of binding, White et al. devised so-called 'toggle' selections (9). Aptamers against thrombin were selected in which target protein was 'toggled' from human to porcine between individual rounds. Since the resultant aptamers could bind both targets, they could be used both for animal and clinical trials. In contrast, negative selections can be incorporated during the selection process to greatly reduce affinity for even closely related targets. Using negative selection, aptamers have been selected that can discriminate between phosphorylated and unphosphorylated proteins (10).

Aptamers can also be engineered for different sites of localization and expression. While antibodies are typically directed against extracellular targets, aptamers can be expressed from within the cell in a gene-therapy approach, termed 'intramer' therapy (reviewed in (2, 11, 12)). By modulating the levels of intracellular expression it may be possible to adapt the amounts of intramer therapeutics produced to differing amounts of a target protein at different stages of a disease or in different individuals. Aptamers can also be introduced exogenously, in order to modulate the function of targets in the bloodstream or on cell surfaces. Moreover, as reviewed below, various types of signals can be appended to aptamers to direct them to different sites within a cell or to specific organs and tissues (see Section 5.1.3).

Table 1. Examples of medically relevant aptamers

	Target	K_d (nM)	Therapeutic applications	Reference
Anti-viral				
	Rous Sarcoma Virus (RSV)	40	Avian sarcoma	42
	Hepatitis C NS3	10	Viral replication	31-33
	Human Cytomegalovirus	35[1]	Viral replication	43
	Influenza		Viral replication	40
	HIV-1 reverse transcriptase	1	HIV replication	7, 25
	HIV-1 integrase	10	HIV replication	199
	HIV-1 Rev	<1	HIV replication	200
	HIV-1 nucleocapsid	2.3	HIV replication	201
	HIV-1 Tat	0.1	HIV replication	202, 203
Cell growth & tumorigenesis				
	alpha-thrombin	25	Thrombosis	98, 204, 205
	Activated protein C	110	Thrombosis	206
	Protein tyrosine phosphatase (PTPase)	18	Oncogenesis, viral regulation	207
	beta-2-integrin		Cell adhesion	81
	Nuclear factor kappa B (NF-kappa-B)	1	Apoptosis	88
	Extracellular signal-regulated kinase 2 (ERK2)	1-5	Cell overproliferation	10
	human TNF-α	n/a	Regulation of immune defense	208
	Basic fibroblast growth factor (bFGF)	0.35	Angiogenesis	55
	Keratinocyte growth factor (KGF)	0.0003	Epithelial hyperproliferative disease	209
	Platelet derived growth factor (PDGF)	0.1	Tumor development	210
	Vascular endothelial growth factor (VEGF)	0.14	Neovascularization	211
	Tumor microvessels		Glioblastoma detection	67
	Tenasin-C	5	Tumor cell detection	70
	Nuclear factor of activated T-cells (NFAT)	10-100	T-cell differentiation and immune response	212
	Wilm's tumor suppressor (WT1)	700	Renal cancer	213
	Prostate specific membrane antigen (PSMA)	20[2]	Prostate cancer	59
	Receptor activator of NF-kappa-B (RANK) receptor	100	Bone malignancy, arthritis	214
	HER3	45	Carcinomas	215
Immune response and inflammation				
	Human neutrophil elastase (hNE)	n/a	Inflammation	118
	Neutrophil elastase	n/a	Inflammatory response	101
	L-selectin	3	Inflammation	216
	P-selectin	0.016-0.71	Inflammatory response	217
	Human non-pancreatic secretory phospholipase A2 (hnps-PLA2)	118	ARDS, septic shock	218
	Immunoglobin E (IgE)	10	Allergies	219
	Interferon gamma (IFN-g)	6.8	Inflammation and immune response	220
	CD4		Immune response	221
	Anti-acetylcholine autoantibodies	60	Myasthenia gravis	73
	Sialyl Lewis X (sLex)	0.085	Cell adhesion; inflammation	78
	Human complement C5	20-40	Immune response	222
	Ku protein	<2	DNA repair, autoimmune disorders, cancer	223

[1] IC_{50}, [2] 2nd order inhibition rate constant

Most importantly, aptamers have yet to demonstrate significant toxicity. The Rossi lab at the City of Hope National Medical Center recently reported interferon response from phage-polymerase-transcribed siRNAs, but delivery of random cellular RNA and synthesized RNA did not trigger a similar response (13). While some nucleoprotein complexes (14) and nucleosomes (15, 16) have been known to induce autoantibody responses in certain individuals, aptamers do not seem to be immunogenic, by and large. Experiments designed to test the cytopathic and toxic effects of nucleic acid therapies so far show no side effects. Kohn *et al*. transduced CD34(+) cells from pediatric patients with aptamer-length decoys of the HIV Rev-responsive element and introduced the transduced cells into patients. No toxic effects were seen, and the transduced genes were still detectable, albeit at very low levels, a year after infusion (17). More recently, anti-VEGF aptamers successfully passed clinical trials for treatment of macular degeneration without any notable toxicity and have since received FDA approval (Eyetech Pharmaceuticals and Pzifer, Inc., 2004).

4. APTAMERS AS THERAPEUTICS

The adaptable nature of aptamers has made it possible to target a number of pathogens that have hitherto proven elusive. In many instances, multiple viral targets whose variability might otherwise overwhelm the immune system can be targeted by multiple aptamers in parallel. The ability to target whole cells as well as protein targets may allow aptamers to be 'blindly' developed against pathogens and transformed cells. And the fact that aptamers appear to have low immunogenicity and toxicity may make them especially useful in combating diseases related to the immune system and inflammation.

4.1. Aptamers against viruses and pathogens

Therapeutic targets that derive from parasites that attack human hosts are perhaps the easiest to identify (although not necessarily the most efficacious, given the defenses of many parasites). In this regard, aptamers have now been raised against a number of viral and pathogen proteins, and are being further developed as therapeutics.

Viruses, especially those with a high frequency of mutation, present a unique problem for the immune system. Many viruses, such as HIV and influenza virus, generate too much variability for the immune system to subdue either in a single infection or over an individual's lifetime. Reservoirs of these viruses often exist as extremely degenerate 'quasispecies.' For example 9% variation between Hepatitis C virions can exist within a single host (18).

Many current treatments for highly mutable viruses have sufficed in prolonging the lives of afflicted individuals. But no single treatment has yet overcome the problem of viral heterogeneity. To generate barriers against mutation, multiple drugs can be administered serially or in parallel, in combination therapies. However, each new organic drug introduced into an individual must be separately advanced through clinical trials, and then tested for cross-reactivity with the other, equally idiosyncratic therapeutics. A great advantage of aptamers is that their salient pharmacokinetic characteristics are likely to be uniform, and thus the development of combination therapies may prove to be as simple as the generation of a new aptamer against a different viral epitope or target. Such combination therapies could potentially involve aptamers that targeted all phases of a viral life cycle. One set of aptamers could be directed against specific extracellular epitopes that might otherwise be shielded from the significantly larger components of the immune system. Another set of aptamers against intracellular targets could potentially address the treatment of latent reservoirs, such as those seen with HIV.

Some of the earliest antiviral aptamers were directed against HIV. Aptamers have been selected against a number of HIV proteins (Table 1), and most of these aptamers have also shown antiviral efficacy in infected tissue culture cells. For example, anti-RT aptamers were able to reduce HIV infectivity 90 – 99.5% in 293T cells (19). Anti-Rev aptamers were shown to effectively subdue HIV infection in several tissue culture lines (20-22). In an experiment that presages gene therapy, an anti-Rev aptamer expression construct was transduced into CD34(+) cells and the differentiated cells were significantly more resistant to HIV infection than control cells (23). The fact that there are multiple aptamers against multiple targets may allow combinatorial aptamer therapies to be developed. Just as the current HAART treatments forestall the evolution of resistant virus, multiple aptamers should also be 'resistant to resistance.' Fisher *et al.* screened a library of mutant RTs for enzymes that would be resistant to the anti-RT aptamer RT1t49 (24, 25). Two mutations in RT were isolated that conferred resistance to the aptamer; however, the resistant RTs were significantly less capable of replication and showed no detectable infectivity after three weeks in tissue culture. Due to the HIV-focused content of these reviews, however, this chapter will focus primarily on non-HIV-related aptamer therapies.

4.1.1. Hepatitis C Virus

The hepatitis C virus (HCV) chronically infects an estimated 2.7 million people in the United States and almost 300 million world-wide (26, 27). Chronic infection by HCV can lead to hepatitis, cirrhosis and liver carcinoma. The current treatment for HCV typically consists of interferon and the nucleoside ribavirin. However, many patients fail to respond to this treatment, and approximately 75% of treated individuals show adverse side effects broadly ranging from fatigue to anemia to suicidal tendencies and depression (28), reviewed in (27). Aside from a high genetic variability that can baffle the immune system, HCV can also ~~i~~nfect B- and T-lymphocytes as well as hepatocytes, thereby disabling immune cells directly (29). Given these effects, an 'extra-immune' biopolymer such as an aptamer might be an especially effective treatment.

The HCV genome consists of a single, positive-stranded RNA that encodes a large polyprotein. The polyprotein is comprised of all the viral proteins and is processed by host factors and two viral proteases, NS2-3 and NS3 (29). Stopping the translation or processing of this polyprotein should severely limit HCV infection. To this end, the Nishikawa group at the National Institute of Advanced Industrial Science and Technology in Japan has generated aptamers against various portions of the NS3 protein (30-33). Aptamers against just the protease domain, for example, bound with an apparent K_d of 10 nM. At a 5-fold molar excess (3.5 micromolar) of aptamer over target protein, the aptamers inhibited protease activity *in vitro* by up to 90%.

The Nishiskawa group has further engineered the anti-NS3 aptamers to bolster their expression levels and efficacy in tissue culture, and thereby their potential therapeutic utility. Aptamers were cloned into stem IV of the Hepatitis delta virus (HDV) ribozyme, and HDV-aptamers chimeras were expressed as concatamers from a chicken beta-globin (CAG) promoter. An additional HDV ribozyme was later added to the 3' end of the aptamer construct to remove 3' poly(A) sequences. In principle, the cis-acting HDV ribozymes should cleave away the aptamers from the body of the mRNA to ensure unencumbered folding. The HDV-aptamer constructs inhibited NS3 protease activity by up to 50% in tissue cultured liver cells; inhibition increased proportionately to the number of concatamers expressed (34). It should be noted that Joshi and Prasad had previously also used hammerhead ribozymes to release HIV inhibitory aptamers from a longer mRNA (19). Because of their small size and tight binding affinity, aptamers were able to bind to and be co-packaged with RT. The packaged anti-RT constructs thus stopped subsequent rounds of infection, and were able to reduce the overall infectivity of a number of different viral isolates.

More recently, Fukuda *et al.* appended a poly-14-U tail to the 3' end of a minimized version of their NS3 protease aptamer (31, 35). Such poly(U) sequences had previously been shown to bind to the helicase portion of NS3 (36). The resulting construct demonstrated stronger inhibition of both protease and helicase activity than either of the single components. The aptamers maintained their specificity and inhibitory effect while tolerating

modifications to their 3' ends. This again demonstrates the extremely adaptable nature of aptamer drugs relative to organic reagents or antibodies. Similar, modular modifications of these other drug types would have been more difficult to accomplish, and would have more likely rendered the original drugs inactive or ineffective.

Aptamers have also been selected against a conserved internal ribosome entry site (IRES) in the 5' UTR of HCV (37, 38). Biotinylated DNA probes complementary to a small section of domain II of the IRES were tethered to streptdavidin beads on a surface. An RNA pool was then incubated with the probe-bound domain II, and unbound species were washed away. Binding of each round was monitored in real-time using surface plasmon resonance. The best binders were in the low nM range and inhibited *in vitro* IRES-dependent translation (38). This demonstrates that aptamer drugs could potentially be launched against both protein and non-protein targets.

Based on this body of work alone, an anti-HCV intramer therapy could be envisioned that targeted three separate viral activities (protease, helicase, and IRES), and that might be included on a single, internally processed transcript.

4.1.2. Influenza Virus

The influenza virus remains incurable despite of decades of research. An estimated 36,000 people die each year from influenza in the United States (Centers for Disease Control: http://www.cdc.gov/flu/about/disease.htm). While flu shots are available to prevent infection, their effectiveness rests on health workers' abilities to predict the specific strains of influenza that will be circulating. The ability to craft biopolymer drugs that would be more broadly antagonistic to multiple different strains of the virus would therefore be very desirable.

The influenza virus presents two important membrane surface glycoproteins that can be targeted by the immune system: hemagglutinin (HA) and neuraminidase (N). The combinations of variants of the two proteins produce different, and sometimes phenotypically unpredictable, strains of influenza. HA plays a key role in initiating infection and is highly variable. The receptor binding site is relatively conserved; however, it resides within a pocket that is not likely exposed to the immune system (39, 40). Therefore, vaccines are directed against the variable portions of the surface proteins and must be adjusted each year.

It is therefore notable that aptamers were selected that could access the receptor-binding pocket. Jeon *et al.* identified a conserved peptide segment (HA91-261) of the receptor binding site in HA which, though not normally exposed on the virus, could readily elicit immune response when cloned and expressed in mice as a peptide (40). DNA aptamers were selected against the HA peptide. At 50 pM, the anti-peptide aptamers were able to reduce mortality of tissue culture cells infected with 500 $TCID_{50}$ by 80%. In mouse studies, aptamers (125 pmole/mouse) were introduced intranasally one day before, the day of, or two days after infection w/ 2,500 $TCID_{50}$ influenza virus. Over two weeks, the body weights of the infected mice were monitored. Untreated mice lost about 20% of their body weight while treated mice displayed minimal weight loss and showed faster recovery. Histological examination of lung tissue also showed that lung viral titer was 1.2 log lower in mice treated before or after infection and 2.4 log lower in mice treated with aptamers coincident with infection. In other words, aptamers were able to inhibit infection both therapeutically and prophylactically. Importantly, aptamers were able to inhibit three different strains of the virus (41).

4.1.3. Whole virus inhibition (RSV & HCMV)

Aptamers have also been selected against whole viruses, circumventing the need to identify or purify individual proteins and providing a more native context for target recognition. In the first demonstration, aptamers were selected against whole Rous Sarcoma Virus (RSV). Treatment of RSV with 20 nM of the selected aptamer reduced viral protein production in infected quail cells by ~90%, while 160 nM aptamer obliterated protein expression. Selected aptamers were able to completely neutralize RSV particles without adverse effects on the host cell. At higher concentrations (15-20 fold), the aptamers were also able to inhibit a different strain of RSV whose surface glycoprotein shared ~95% homology with the strain used in the selections (42). However, in these experiments virus was first mixed with aptamer prior to treatment, a situation unlikely to actually occur if the aptamer were used as a therapeutic. In addition, preincubation of quail cells with 500 nM RNA for an hour, followed by washing did not appear to reduce infection. Thus the aptamers appear to be acting transiently as extracellular inhibitors of viral entry.

Aptamers have also been selected against human cytomegalovirus (HCMV). A number of high-affinity aptamers were isolated from the selection, but only a few families proved useful in treating HCMV infection**.** Since the surface proteins of HCMV have not been fully characterized, those aptamers not inhibiting HCMV may represent aptamers binding to other surface proteins not involved in receptor recognition or infection. When HCMV was treated with either of two aptamers, L13 or L19, and then applied to human foreskin fibroblasts, the viral titer was significantly reduced. An IC_{50} of approximately 100 nM and 35 nM was observed for L13 and L19, respectively. As with the anti-RSV aptamers, the anti-HCMV aptamers were added to the virus prior to infection. The two aptamers did not compete for binding, suggesting that they acted at different sites on the virus. Crosslinking experiments showed that the aptamers bound to gB and gH, two HCMV surface glycoproteins important for viral entry and infection. While the aptamers were able to inhibit two different strains of HCMV, inhibition was specific for HCMV, as the aptamers had no effect on herpes simplex virus-1 (HSV1) which displays homologous gB and gH proteins (43).

4.1.4. Aptamers against other pathogens

Many parasites have evolved mechanisms to evade the human immune system. Drugs against parasites

are limited, and because of complications with long-term toxicity and the emergence of resistant parasites, the available treatment of parasitic diseases needs to be reassessed (44, 45). Several researchers have begun to develop aptamers against parasitic targets. While these aptamers are not yet therapeutically useful, the selection experiments have nonetheless provided some insight into parasitic mechanisms.

RNA aptamers have been selected against live African *Trypanosoma brucei*, the protozoan that causes African sleeping sickness. The aptamers bound within a flagellar pocket with a K_d of 60 nM (8). The selection was intriguing not only because it was against live, whole cells, but also because the aptamers recognized the organism in a manner different than antibodies. While the variability of the surface glycoproteins (VSGs) that cover Trypanosomes frequently confounds immune responsivity , the aptamers were able to recognize the protein in multiple strains of the protozoan, including strains that were not used for the selection experiments.

In follow-up work, Homann and Goringer showed that aptamers binding at the flagellar pocket were eventually taken in by an endocytotic process and transported to the lysosome. Transport appeared to be receptor-mediated and sequence specific, requiring a minimal core aptamer sequence. Most importantly, the authors monitored transport using a biotin conjugate; these results suggest that aptamer conjugates might be used to direct biotoxic compounds directly into trypanosome cells (46).

Another selection by the same group produced aptamers against the variable surface glycoprotein (VSG) itself (47). Since VSGs are inherently variable, the initial pool was incubated with a soluble VSG (sVSG) preparation to first enrich for VSG binders. Then bound RNAs were re-isolated and incubated with live cells expressing the same VSG variant in order to select for RNAs capable of binding VSG within the context of the complex cell surface. In later rounds, different sVSG preparations and antigenically variant clones of Trypanosomes were used. Aptamers were selected that could bind in the subnanomolar to nanomolar range to the different sVSG variants used in the selection. Aptamer cl.57 bound to two glycoprotein variants, sVSG 221 and sVSG 117 with K_d's of 0.3 nM and 0.4 nM, respectively. The aptamer also bound to two other sVSGs that had not been included as targets with similar K_d's (0.67 nM and 0.72 nM). These results again suggested that aptamers could readily recognize conserved sequences or structures between VSG molecules.

As with the aptamers selected against whole organisms, biotin tags attached to aptamers did not significantly disrupt binding of the anti-sVSG aptamers, and distribution of the VSG layer could be imaged using fluorescently labeled streptavidin. Substituting fluorescently labeled anti-biotin antibodies instead of streptdavidin, Lorger *et al.* showed that antigen-conjugated aptamers could attract antibodies to the Trypanosome surface (47). Such a mechanism could potentially be used to direct antibodies to cell components that might otherwise be masked to immune response.

In another approach, Ulrich *et al.* set out to select for aptamers that would disrupt the cell-surface interactions between *Trypanosoma cruzi* and its host (48). Evidence has suggested that *T. cruzi*, as well as several other parasites, recognize and bind to several host cell surface proteins during infection: fibronectin, laminin, heparin sulfate and possibly others. Ulrich *et al.* recognized that certain parasitic epitopes were only expressed on the surfaces of infective trypansomes (trypomastigotes), and not on the non-infective stages (epimastigotes). These authors posited that the infective-stage proteins might be involved in interactions with the host cell proteins. In order to select against infectivity-specific surface molecules on trypomastigotes, a negative selection was first carried out using non-infective stages of *T. cruzi* followed by selection against infective *T. cruzi*. Aptamers that bound specifically to trypomastigotes were isolated and divided into classes based on which host protein could best displace the aptamer:trypomastigote interaction: heparin, fibronectin, thrombospondin, or laminin. While the other proteins have been shown to interact with *T. cruzi*, these experiments provided evidence that thrombospondin may also be involved in *T. cruzi* infection interactions, and thus serve as an example of how aptamers can be used to dissect the role of the host in infectivity. Aptamers formed complexes with the organisms that had dissociation constants in the range of 40 nM to 400 nM, and at 1 micromolar could inhibit trypanosome infection by about 50 – 70%. Although binding and therapeutic efficacy were modest, these results bode well for the further development of aptamers that can inhibit parasite:host interactions.

4.2. Aptamers in cancer treatment

At root, immune recognition is about biopolymer recognition, and biopolymer recognition is about specificity. One of the greatest specificity challenges facing the immune system is the ability to distinguish subtle but pathological alterations in metabolism and physiology; for example, cellular transformation and oncogenesis. The ability to discern a cancerous cell from normal epithelial tissue therefore represents a challenge that aptamer therapeutics are uniquely suited for. Aptamers have been generated against a number of tumor-related targets. In addition, aptamers can be raised *de novo* against transformed cells and tissues without a prior knowledge of a relevant target.

4.2.1. Aptamers against tumor antigens

The unregulated proliferation of cancerous cells is assisted by any of a variety of proteins whose form or expression differs from that seen in a healthy individual. Such tumor antigens are natural targets for the identification of therapeutic interventions, whether drug or biopolymer.

One notable example of a mis-expressed protein is vascular endothelial growth factor (VEGF), which contributes to neovascularization and vascular permeability

of solid tumors. A number of anti-VEGF aptamers have been selected and bind to the protein with low nanomolar to picomolar affinities (49, 50). Aptamers against VEGF have proven effective in inhibiting tumor proliferation in multiple cancer types. Wilms tumors induced in nude mice underwent 84% weight reduction when treated with daily intraperitoneal (i.p.) injections of 200 micrograms of an anti-VEGF aptamer for 5 weeks. None of the aptamer-treated mice (n = 10) showed adverse effects compared to control (51). Similarly, human neuroblastoma tumors xenografted into mice exhibited a 52% reduction in growth after daily i.p. injections of 250 micrograms aptamer for five weeks. The tumor reduction was on par with an anti-VEGF antibody, but lower than a previously designed inhibitor composed of VEGF decoy receptors attached to an antibody segment (52, 53). Further development of anti-VEGF aptamers has allowed them to pass human clinical trials, although so far for age-related macular degeneration rather than for cancer. The anti-VEGF aptamers may also eventually prove useful against other diseases, such as psoriasis.

Other growth factors have also been targeted for reduction of tumors. Basic fibroblast growth factor (bFGF) is a cytokine often associated with wound healing and whose expression affects cell proliferation, angiogenesis and tumor spread. Serum levels of bFGF have been statistically shown to be higher in breast cancer patients than non-cancerous patients (54). Jellinek *et al.* selected aptamers against bFGF that could form complexes with K_d values as low as 3 nM (55). More recently, Golden *et al.* (56) used a modified selection scheme called photo-SELEX (57, 58) to select even tighter-binding anti-bFGF aptamers. In photo-SELEX, random pools are synthesized with a photosensitive nucleotide, such as 5-bromo-2'-deoxyuridine (BrdU). Following incubation with a target, the mixture is irradiated with 308 nm light. At this wavelength, BrdU cross-links with proximal aromatic and sulfurhydryl moieties on amino acids. The cross-linked sequences can be readily separated from non-binding species and re-amplified. The photo-SELEX derived aptamers bound with K_d values as low as 16 pM, did not significantly bind to VEGF or PDGF, and retained their binding affinity even in 10% serum.

Many tumor antigens are membrane-associated, and and a number of cell surface proteins have served as aptamer targets (Table 1). Lupold *et al.* (59) have selected aptamers against the well-known prostate-specific membrane antigen (PSMA). PSMA is a hydrolase whose physiological function is unclear, but it is overexpressed on the surface of at least some prostate tumors. By using a recombinant variant of prostate-specific membrane antigen (xPSM) these researchers were able to isolate two aptamers, xPSM-A9 and –A10. Both aptamers inhibited xPMS activity with K_is in the low nanomolar range. Both A9 and A10 were shown to bind a PSMA-expressing prostate tumor line, LNCaP, but not to a different prostate tumor line, PC3, which does not highly express the protein.

Aptamers need not serve as therapeutics in their own right, but can potentially be used to target the delivery of other drugs. In an elegant set of experiments, Farokhzad *et al.* (60) incorporated Lupold's A10 aptamer into nanoparticle-aptamer bioconjugates and effectively transported dextran (a model drug) to prostate cancer cells. There was a 77-fold increase in uptake by aptamer-conjugated particles over controls as seen by fluorescence labeling.

Aside from potentially acting as therapeutics, aptamers against tumor antigens could be used to diagnose disease states. For example, photo-SELEX derived aptamers have been mounted in microarray-like formats. Smith *et al.* (61) first proofed this technology by mounting anti-bFGF and -$gp120_{MN}$ photoaptamers onto N-hydroxysuccinimide-activated slides and detecting the presence of these proteins in serum solutions using NHS-Cy3 reactive dyes. Sample serum was applied to the array, and individual proteins were crosslinked to the aptamers. After washing, aptamer-linked proteins could be detected using fluorescent dyes. Two anti-bFGF aptamers were tested and could detect bFGF in the picomolar range, six orders of magnitude better than to the closely related aFGF. Similarly, aptamers against $gp120_{MN}$ could detect this protein in the nanomolar range, four orders of magnitude better than gp120 from a different HIV strain, SF2. Bock *et al.* were later able to quantitatively detect 17 different targets in 10% serum using a single photoaptamer array (62). Aptamers were detected using either using universal protein stains or labeled target-specific antibodies in a format the authors termed an antibody-linked oligonucleotide assay (ALONA).

4.2.2. Aptamers against whole cells

An inherent problem with cancerous cells is that they often do not adequately trigger a response from the immune system. A number of possible mechanisms for this evasion have been proposed, and these may act in combination. Tumor cells may down-regulate tumor-associated-antigens (TAAs), express immunosuppressive cytokines or just fail to produce TAAs significantly different from 'self' cells (reviewed in (63)).

Therefore, it would be useful if aptamers could detect slight changes in cell surface expression, especially changes that might elude immune surveillance (as with sVSG). Selections against cells can specifically target a phenotype, rather than an individual protein, and thus obviate the need to identify and purify tumor antigens. Moreover, there exists the possibility that purified targets may not behave in the same manner as they do in the context of the cell surface. It should be noted that the utility of whole cell selections has previously been exploited using phage-display technologies (reviewed in (64, 65)).

In an early step toward whole cell selections, Morris *et al.* (66) showed that aptamers selected against a cell can have similar binding affinities to those raised against purified protein targets. Selections against human red blood cell (RBC) ghosts produced a diverse pool of selected aptamers. Two motifs that appeared with some frequency (16% and 10% of the entire pool) were further

analyzed. These binding analyses yielded affinities in the low nanomolar range (1 to 2 nM). Using a procedure they termed 'deconvolution selection', these researchers were able to identify the cell surface targets more definitively. Selected aptamers were conjugated to photoreactive phenyl azide groups, incubated with RBC ghosts and then irradiated. Four major cross-linked products were excised from polyacrylamide gels, re-amplified, and the procedure was repeated. After four rounds, four individual selected pools corresponding to tight binding of each of the four major cross-linked products were isolated.

These techniques or variations on them have now found use in the identification of other anti-tumor aptamers. Transformed rat YPEN-1 endothelial cells (EC) were used as targets to generate ssDNA aptamers against glioblastoma (67). Starting with a counter-selection against murine N9 microglial cells, aptamers were selected that specifically bound microvessels of rat glioblastomas but not normal rat brain tissue or peritumoral areas as measured by flow cytometry and fluorescence microscopy. The authors also performed a 'scratch test' (damaging of a monolayer) with cultured endothelial cells. One high-affinity aptamer, III.1, localized to the scratch following application, suggesting that the aptamer target might be involved in proliferation or cell repair. The aptamer was thereafter also employed for target identification. Affinity purification of the cross-linked aptamer-target complex and peptide mass spectrometry identified the aptamer target as the rat homologue of the mouse protein pigpen. Consonant with the scratch test observations, inhibition of pigpen has recently been shown to inhibit angiogenesis and proliferation in EC (68).

Similarly, ssDNA ligands were selected against differentiated PC12 cells and negatively selected against the corresponding parental cell line (69). During the subtractive step, the pool DNA was exposed to undifferentiated cells, and any binders were eliminated. When presented with a 1:1 mixture of differentiated and normal cells, the aptamers bound specifically to the differentiated variety; no binding to undifferentiated cells was observed. The best aptamer bound target cells 100-fold better than a control oligonucleotide, as determined by fluorescence quantitation with FITC-labeled ligands. Like previous phage display selections, these two sets of experiments showed that tumor-cell specific aptamers could be selected simply by incubating pools with pathological cells while subtracting the background of normal cells.

In a somewhat more elaborate scheme, Hicke *et al.* (70) selected anti-tenascin-C (TN-C) aptamers by selecting against both U251 glioblastoma cells and purified TN-C protein. TN-C is an extracellular protein involved in tumorigenesis, wound response, embryogenesis and proliferation. Its re-expression later in life usually signals tumorigenesis or other pathology, and levels have been shown to be much higher in tumors than in normal cells. A previous selection had been performed against U251 cell monolayers (71), and one of the selected aptamers was shown through affinity purification to bind to TN-C (K_d of 150 nM). However, in order to prevent receptor mediated internalization of aptamers, the selections had been performed at 4° C. When tested at 37° C, the K_d was at least 10-fold worse. Moreover, the selected aptamers were single-stranded DNAs without any stabilization features. With this prior knowledge, Hicke *et al.* designed different selection schemes to isolate higher affinity aptamers and also compared methods for generating aptamers against tumor cells. Three selections using 2'-fluoro-pyrimidines were carried out: against purified TN-C, against whole U251 cells in which aptamers were eluted with either EDTA or Trizol, and a cross-over selection in which aptamers were first selected against whole cells, and then subjected to two more rounds with purified protein target. In general, the authors found that EDTA was the best choice for elution; Trizol resulted in background RNAs being carried through the selection. The purified protein selections gave rise to aptamers with K_d values of 3 nM, whole cell selections produced aptamers with K_d values of 100 nM, and the cross-over selections gave K_d values similar to purified target (2 nM).

That this wide variety of techniques all served to identify aptamers specific for tumor cells is a strong validation of the general potential of selection experiments to produce useful anti-tumor aptamers. With such binding reagents in hand, diagnostic, therapeutic, or targeting applications that distinguish cancerous cells relative to normal epithelial tissues can now be envisioned and developed.

4.3. Aptamers and immune disorders

Aptamers may be particularly well-suited for treating disorders of the immune system. Treatment for immune disorders commonly involves immunosuppression drugs. Not only are toxic side effects an issue, patient health is compromised by having a weakened immune system. The demonstrated specificity of aptamers, as well as their apparent lack of immunogenicity and toxicity, argue that aptamer therapies would not similarly disrupt the system.

Indeed, instead of weakening the immue system, aptamers can potentially be used to temper culprit antibodies. In an early display of this potential, Tsai and Keene (72) performed a selection against serum from a patient with systemic lupus erythematosus (SLE). SLE patients produce anti-DNA autoantibodies leading to autoimmune damage in many organs. The mechanism of the autoimmunogenicity is not clear, and the types and structures of nucleic acids recognized by these antibodies have not been fully elucidated. Interestingly, the aptamers selected by Tsai and Keene resembled portions of U1 small nuclear RNA (snRNA) that had been shown to react with autoantibodies. These authors further showed that their aptamers could inhibit an autoantibody reaction caused by U1 snRNA.

Selections relevant to the neuromuscular disease myasthenia gravis (MG) have also been carried out. In MG, autoantibodies down-modulate nicotinic acetylcholine receptors (AChRs) on the surface of skeletal muscle cells.

Lee and Sullenger (73) selected aptamers against a rat anti-AChR antibody, mAb198, and the resultant complex had a K_d of 60 nM. Hwang *et al.* (74) then re-selected for aptamers with up to 10-fold stronger binding than the previously selected aptamers. At a concentration of 3 micromolar, the anti-AChR aptamer, Ex-SE-RNA, was able to inhibit mAb198 antibody-induced down-modulation by up to 80%. A slightly reduced activity was seen when assayed with antibodies from patients with MG in place of mAb198. Ex-SE-RNA was able to inhibit autoantibodies from MG patient serum in a dose-dependent manner with an IC_{50} of 2 micromolar. In later work, anti-AChR aptamers were truncated and attached to PEG moieties for better *in vivo* retention without disruption of the inhibitory effect (75).

An MG-like autoimmune state, called experimental autoimmune myasthenia gravis (EAMG), can be induced in animals by injection with AChR or transfer of anti-AChR antibodies (such as mAB198). Hwang *et al.* (75) tested the ability of their truncated and PEGylated aptamer to alleviate EAMG in rats. In non-aptamer-treated animals, muscular AChR content was reduced by 51% and muscular weakness manifested within 24 hours. Animals treated with aptamer showed only approximately 17% AChR loss. Aptamers may therefore also prove useful in the treatment of EAMG. To the extent that different antibodies might arise in either MG or EAMG than those that have currently been selected against, new aptamers could nonetheless readily be generated using whole serum or purified antibodies, even on a patient-by-patient basis. Such individually crafted therapeutics may not be currently cost-effective, but with the discovery of allele-specific diseases and drug effects, modern medicine is increasingly focused on the individual. To the extent that individual-specific aptamers can be more readily identified, produced, and delivered than individual-specific organic compounds, aptamers may represent an important addition to this trend.

Of course, immune disorders are not limited to severe autoimmune reactions. Inflammation can lead to chronic ailments that include arthritis, myocarditis, nephritis, and colitis. Anti-inflammatories, corticosteroids, and other medications can address the symptoms of these diseases, but they are not treatments per se, and long term use can lead to undesirable side effects. Again, aptamers may enable specific factors involved in inflammation to be targeted, rather than down-regulating the entire system.

As an example, oncostatin M (OSM) is a cytokine strongly implicated in rheumatoid arthritis and may have roles in mediating cell growth and differentiation. Recent interest in OSM has centered on the demonstration that OSM may inhibit the growth of some melanoma and cancer cells. Obviously, it may be worthwhile to partially or finely inhibit the role of OSM in arthritis without also down-regulating its desirable anti-tumor properties. Aptamers with a K_d of 6 nM have been selected against human OSM. One of the aptamers (ADR58) was able to block binding of OSM to its receptor in a dose-dependent manner with an IC_{50} of 1 micromolar. The aptamers were shown to be specific for human OSM (relative to mouse OSM, 42% identity; TNF-alpha; and the OSM structurally-related leukemia inhibitory factor) and could also specifically block interactions with the OSM receptor (76).

Selectins are another group of surface proteins that may be targets for the development of potent anti-flammatory drugs. Selectins bind to and mediate leukocyte cell adhesion to molecules such as sialyl Lewis X (sLex) on endothelial cells. Subsequently, the leukocytes are transferred (or "rolled") from the initial site of interaction to the site of injury. Selectins are thus integral to the extraction of leukocytes from blood vessels during inflammation, and a disruption of this interaction would essentially interrupt the innate immune response.

Aptamers have been selected against L-selectin (77), which is expressed on leukocytes (as opposed to E- and P-selectins which are inducibly expressed on endothelial cells and platelets, respectively). The anti-selectin aptamers demonstrated nanomolar affinities specifically for L-selectin, and much reduced affinities for P- or E-selectin. Hicke *et al.* (77) also demonstrated inhibition of L-selectin binding to sLeX by their aptamers with an IC_{50} of approximately 3 nM. They further showed that their aptamers could inhibit PBMC "rolling" in an *in vitro*, cell-based assay. In an experimental precursor to *in corpus* delivery, Hicke *et al.* also assayed their aptamer in SCID mice. The anti-selectin aptamers were able to inhibit trafficking of lymphocytes to peripheral and mesenteric lymph nodes as effectively as an antibody control. Biodistribution of lymphocytes throughout the rest of the body, as measured by accumulation of lymphocytes in organs, was not affected.

Targets other than proteins may also prove useful for immune modulation. For example, aptamers have been selected against sialyl Lewis X (sLeX), the extracellular glycan previously mentioned as an L-selectin ligand. Multiple RNA aptamers were generated by 17 rounds of selection against purified sLeX, and apparently exhibited sub-nanomolar to nanomolar affinities, as measured by SPR (surface plasmon resonance). The best clone (K_d = 85 pM) bound the glycan target 1000-fold better than did the initial pool RNA. Affinity for sLeX was 5-10 times stronger than for related sugars and 100 times stronger than for a dissimilar sugar, lactose. Inhibition of cell adhesion was demonstrated *in vitro* using a static cell adhesion assay wherein E- or P-selectin-coated wells were incubated with sLeX-expressing promyelocytic leukemia HL60 cells in the presence or absence of aptamers, and the number of adhered cells was counted (78).

5. APTAMERS IN BIOMEDICAL RESEARCH

While some aptamers have clear therapeutic utility at present, others are more useful as tools for dissecting and understanding biology. Nonetheless, an examination of these aptamers and their effects is useful in that they provide examples of what other therapeutic applications there may be for aptamers. For example, to the extent that aptamers can specifically disrupt or

modulate signal transduction, then it may be possible for aptamers to influence pathologies that involve a failure in signal transduction mechanisms, including many tumors.

5.1. Signaling pathways and cell regulation

Signaling and cell-cycle pathways present ideal fields for aptamer use. Figuring out a protein's role in the cell, as an example, often requires finding appropriate mutants or small molecules that can inhibit the protein. However, players in a pathway may be closely related to other factors, have no specific inhibitors or may not be conducive to mutational studies. Aptamers can be selected to specifically knock down one link without altering the genetic makeup of the cell or interfering with any other cofactor. In addition, it should be possible to express aptamers intracellularly to block signal transduction targets; in contrast, many disulfide-laden antibodies will not work well within the reducing environment of the cell.

A good example of an aptamer that can be used for intervention in signal transduction networks is the aptamer selected against the protein kinase ERK2. ERK1 and ERK2 (ERK1/2) are members of the mitogen-activated protein (MAP) kinase family. MAP kinase pathways are involved in signaling cell growth, apoptosis, differentiation, cancer, autoimmune diseases, cell stress and possibly other undefined roles (79). Understanding the pathway (and identifying reagents for modulating the pathway) would, therefore, be beneficial, but the MAP kinase family consists of many proteins that are too closely related to allow the easy identification of specific organic inhibitors. However, Seiwert *et al.* selected against the activation lip of ERK2 and found aptamers that could specifically inhibit ERK1/2 in the low nanomolar range, but that do not inhibit the related JNK2 or p38 proteins. Interestingly, one family of selected aptamers was able to distinguish between the phosphorylated and unphosporylated forms of ERK2 with a preference for the phosphorylated form (10).

Similarly, *in vitro* selection was used to find specific inhibitors of the Ras/Raf-1 interaction. Raf-1 is a serine/threonine protein kinase that activates the MAP kinases. Like MAP kinases, inhibitors of the Ras/Raf-1 interaction have been difficult to find due to the fact that there are a large number of closely related proteins. Kimoto *et al.* carried out a selection against the Ras binding domain of Raf-1 (RBD). The resultant aptamers inhibited the interaction between Ras and Raf-1 with K_d values of between 150 and 360 nM. However, the aptamers did not bind to the Raf-1 isozyme, B-Raf, or to another Ras partner, RGL (80).

While these examples demonstrate the power of *in vitro* selection for generating specific binding reagents, the Famulok lab at the University of Bonn in Germany has pioneered the use of intramers for modulating and dissecting signal transduction pathways. Blind *et al.* targeted beta-2-integrin LFA-1 (CD11a/CD18), a transmembrane protein responsible for cell adhesion to intercellular adhesion molecule-1 (ICAM-1). Expression of the aptamer in Jurkat cells was able to inhibit cellular adhesion. By carrying out selections against peptide fragments of the cytoplasmic portion of CD18, Blind *et al.* were able to demonstrate intracellular control of extracellular interactions. Defining the components of this pathway may also yield insights into immune and inflammatory responses (81). Similarly, Mayer and Famulok selected aptamers against recombinant cytohesin-1, a guanine nucleotide exchange factor (GEF) for ADP – ribosylation factor (ARF) GTPases. Not only did the authors show that cytohesin-1 played a role in integrin-mediated cell adhesion, they also found that cytohesin assisted in actin cytoskeleton reorganization (82).

5.2. Gene expression

It is no surprise that aptamers can be raised against nucleic acid binding proteins, and therefore that intramers may also be used to precisely control gene expression. Indeed, one of the earliest selections was targeted against the T4 DNA polymerase (83). Thomas *et al.* later selected for aptamers against yeast RNA polymerase II (Pol II). The aptamers bound *Saccharomyces cerevisiae* Pol II with a K_d of 20 nM, but did not bind Pol I or Pol III, nor did it inhibit Pol II from the related *Schizosaccharomyces pombe* or from wheat germ cells (84).

In addition to polymerases (including reverse transcriptases, as described above), aptamers can be raised against regulatory proteins involved in transcription. NF-kappa-B is a human transcription factor involved in a wide array of cell responses including: inflammation, apoptosis regulation, HIV transcription and more (85). Inhibition of NF-kappa-B has shown reduced damage to heart tissue after oxygen starvation (86) and stimulation of tumor cell killing in cancer therapy (87). Lebruska and Maher selected RNA aptamers against the NF-kappa-B homodimer, binding with an affinity of approximately 1 nM (88). Cassiday and Maher demonstrated *in vivo* binding of the selected aptamer, alpha-p50, to NF-kappa-B using a yeast three-hybrid system (89).

Recently, Cassiday and Maher revisited the yeast three-hybrid assay to explore different ways of isolating even more potent aptamers. Using a degenerate, or doped, pool based on their previously selected aptamer, alpha-p50, Cassiday and Maher performed an *in vivo* selection with the yeast three-hybrid system and isolated aptamers that produced 3-fold higher beta-galactosidase expression than the original parental aptamer. The majority of sequence differences occurred outside of the region previously shown to be important for binding. To investigate whether tight-binding aptamers might have been lost during the selection, Cassiday and Maher used the assay to also screen an earlier round, Round 8. One sequence, RNA 6, was identified that enhanced reporter expression 10- to 20-fold. Interestingly, the differences in *in vitro* binding affinities between alpha-50 and RNA 6 were indistinguishable (90). Unsurprisingly, aptamers selected *in vitro* may yet behave differently *in vivo*, and vice versa.

Another example involves the B52 protein (also SRp55), a regulator of mRNA splicing and an important protein in *Drosophila* development. Aptamers against this

protein were selected that bound in the nanomolar range (91). In an effort to boost the aptamer's effects, Shi *et al.* designed a so-called inhibitory aptamer RNA (iaRNA). Each iaRNA consisted of five aptamers cloned together to form a pentavalent unit. The pentavalent units, or iaRNAs, were further concatamerized into constructs of 2, 4, 8 and 12 pentavalent units with hammerhead ribozymes between each to release the iaRNA (92). The iaRNA bound B52 10-fold better than monovalent aptamers. Shi *et al*. added a cis-acting hammerhead ribozyme after their iaRNA construct to cleave off polyadenylation signals, a strategy previously shown to retain transcripts in the nucleus (93, 94). Most importantly, the aptamers were expressed in *Drosophila* S2 cultured cells under various promoters to look for highest expression levels. The dodecamer construct expressed under a heat shock promoter was estimated to accumulate to concentrations greater than 100 nM in the nucleus in 10 minutes. The iaRNA expressed under the heat shock promoter was transformed into *Drosophila* germ lines and immunofluorescence studies showed strong recruitment of B52 to the site of iaRNA expression in fly nuclei.

Having proofed binding of iaRNA to B52, different lines of transgenic flies were made by introducing different iaRNA constructs in tandem with different B52 expression constructs. Typically, overexpression or lack of B52 results in lethality making it hard to perform mutation/inhibition studies. By crossing the transgenic Drosophila lines that expressed varying iaRNA and B52 expression constructs, the authors were able to modulate the levels of aptamer and B52 expression and to thereby witness quite unique phenotypes associated with variable inhibition of B52 expression. For example, B52 overexpression, but not to lethality, caused absence of salivary glands in larva. This and other phenotypes were reversed when the larva co-expressed iaRNA (92). These results provide an excellent example of one way in which aptamers can be used for validating putative drug targets in a way that is at once different and more complete than similar interventions with knockouts, antisense, or siRNA.

6. ADVANCES TOWARD GREATER APTAMER UTILITY

One hurdle that must be overcome before aptamers can be used in a clinical setting is determining how molecules that were originally selected *in vitro* can be adapted to function *in vivo*. Some of the most relevant considerations include how to appropriately deliver aptamers into the body or into cells, how to stabilize aptamers against degradation, how to target aptamers to therapeutically relevant sites, and how to maintain or control aptamer activity or expression. In considering these issues, and as had been alluded to above, there are two major ways to think of aptamer therapeutics: as exogenously delivered drugs and as endogenously delivered gene therapies (or 'intramers'). We will consider the prospects for such treatments separately. However, it should be noted that while exogenous therapeutics will typically target circulating or cell surface proteins and endogenous therapeutics will conversely target intracellular proteins, there is no reason that such therapies cannot be used together, especially against particularly intractable targets, such as viruses.

6.1. Exogenous delivery

Aptamers are, of course, much larger than conventional pharmaceuticals. However, unlike protein therapeutics they can be chemically synthesized in great quantity and modified during synthesis. Thus, aptamers can in some ways be considered to be 'the best of both worlds.' However, significant problems remain with developing aptamer drugs, most notably the method of delivery and stabilization (both physically and in terms of their pharmacokinetic profile).

6.1.1. Injection

Finding appropriate means to deliver aptamers may be one of the largest obstacles to developing aptamers to become therapeutics. Current efforts to deliver aptamers to animal models or patients typically require injection or surgical implantation. While exogenous aptamer delivery is still in its infancy, a variety of nucleic acids have been introduced into human and animal systems by subcutaneous (s.c.; under the skin), intravenous (i.v.; into the bloodstream) intraperitoneal (i.p.; into the abdomen), intravitreal (i.v.t.; into the eye) injection or direct injections into muscle or tissue.

The easiest delivery method relies on simple injection followed by passive transfer into the appropriate tissues; that is, subcutaneous (s.c.), i.p. or direct bolus injections. Nucleic acids delivered this way can ultimately enter the bloodstream or be taken up by nearby cells. As an example, a DNA template that contained the beta-galactosidase gene was introduced using a pressure-based injector. DNA was found in cells up to 2 cm away from the injection site, and beta-galactosidase expression was also seen (95).

However, one disadvantage of s.c., i.p. and direct administration is the vast quanitity of sample needed. Indeed, Liu *et al.* have demonstrated that even larger than conventional volumes of plasmid DNA injected at a faster rate may be more suitable for expression and DNA stability. Ten micrograms of a luciferase expression plasmid in different volumes of saline were injected into mice to determine parameters for optimal expression of exogenous delivery of DNAs. The authors found that a volume of between 80 - 120 mL/kg (e.g. 3 mL plasmid DNA into 30g mice) gave the highest levels of expression, with expression predominately in the liver and then, at much smaller, but evenly distributed levels in the kidney, spleen, heart, and lung. Luciferase expression was also significantly higher when the solution was administered within 5 s compared to 30 s. Expression levels peaked at 8 hrs and declined over the course of several days. Repeated administration reproduced the same expression trends suggesting that subsequent dosing of exogenously delivered genes can make up for lack of long-term expression. However, at the volumes used in these experiments, the injected solution neared the volume of blood in the mice; a proportionately large volume for a human injectable might

limit clinical applications. In addition, while weight and most biochemical markers remained within a normal range over 7 days, alanine aminotransferase (ALT), an enzyme predominantly found in the liver and that is often associated with liver damage, did rise drastically on the first day of injection, but returned to normal within three days (96).

Delivery of aptamers as well as plasmids can lead to systemic effects. For example, anti-PDGF aptamers hold potential as prophylactics and therapeutics for angioplasty, heart bypass or other injury to vessel lumen. An anti-PDGF aptamer was administered intraperitoneally to rats before and after vessel injury and inhibited generalized vessel stress response. A 50% reduction in lesion size at 2 weeks was observed in this study involving 134 animals. Unfortunately, after treatment ended, benefit was lost and vessel lesions worsened (97).

Alternatively, aptamers may be delivered intravenously (i.v.). Delivery by i.v. versus s.c. has the advantages of quick introduction of high concentrations of a therapeutic into the bloodstream and rapid clearance from the bloodstream (see Section 5.1.4. below) which may be preferrable in some applications. For example, for the inhibition of thrombin, a plasma clotting enzyme, effects need to be rapid. Several anti-thrombin aptamers were selected from an N60 random DNA pool. A 15mer truncated version of one aptamer bound with a K_d of approximately 100 nM and increased clotting of fibrinogen almost 7-fold from 25 s to 169 s (98). The aptamer was introduced intravenously during canine heart surgery to prevent clotting. At a minimum dosage of 0.5 mg/kg/min, clotting time rose from 106 s before treatment to 187 s after infusion began and ultimately reached a blood concentration of 11.4 micromolar. The aptamer matched heparin anti-clogging capabilities during surgery. No gross differences were seen between animals treated with heparin compared to those treated with aptamer, and wounds clotted normally after surgery. Interestingly, aptamer half-life was approximately 2 min before and shortly after surgery, but rose to almost 8 minutes during surgery, presumably because blood had been diverted to a heart-lung machine, and organs could not remove aptamers from circulation. The relatively quick clearance after and not during surgery meant no further treatment was necessary to remove the effects of the aptamer once surgery was over. The aptamers had no apparent toxicity allowing for a much broader therapeutic window, unlike heparin, which must be constantly monitored during infusion and which requires antagonists to counteract (99). Similarly, when the anti-thrombin aptamer was administered to cynomolgus monkeys by i.v. at a rate of 0.3mg/kg/min, an anticoagulant effect twice that of a saline control was observed. Linear dose dependence and a short half-life of roughly 100 s were determined (100).

Given some of the problems surrounding systemic delivery, injections at a specific site of action may be best suited for the delivery of nucleic acid aptamers. In this regard, anti-hNE aptamers have been shown to provide localized protection. hNE is a serine protease that participates in degradation of targets in response to various cellular signals. However, uncontrolled or mis-expression of hNE leads to self-proteolytic processes that contribute to vascular breakdown in diseases such as emphysema, cystic fibrosis, acute respiratory distress syndrome and others. A model for rat lung injury can be induced by intrapulmonary application of anti-BSA IgG followed by BSA. The subsequent immune reaction causes lung leakage and hemorrhage, and the level of injury can be correlated to level of lung vascular permeability as measured by leakage of ^{125}I-labeled BSA administered into the bloodstream. In injured rat lung, instillation of an anti-hNE aptamer, NX21909, directly into the intratracheal space inhibited permeability in a dose-dependent manner. At the highest dosage tested (40 nmol), permeability was reduced by 38%. By contrast, 200 nmol of a recombinant protein inhibitor, secretory leukocyte protease inhibitor (SLPI), reduced permeability by 54%. The degree of lung injury can also be inferred by measuring the content of lung myeloperoxidase (MPO), an enzyme often associated with inflammation. NX21909 reduced MPO by 53% compared to 37% by SLPI (101).

As another example of direct injection, intravitreal administration has become popular, as the eye is easily administered to and provides an isolated in vivo site for testing. The anti-VEGF aptamer EYE001 is perhaps the most advanced example of an aptamer therapeutic. When injected into the vitreous humor of rhesus monkeys, EYE001 was detectable and intact 28 days later (102). Studies to determine the pharmokinetic profile of the delivered aptamer, and ultimately to define safe human dosages, used 0.25 – 2.0 mg/eye in a single bilateral dose in addition to biweekly bilateral dosage. Administered in bolus, no toxic effects were observed and no antibody response was elicited in the animals. Anti-VEGF aptamers have now progressed through Phase III clinical trials, delivered directly into the intravitreal space in the eye to control neovascularization that can lead to diseases like macular degeneration. In Phase II clinical trials, 87.5% of patients receiving the aptamer showed stabilized or improved vision three months after aptamer treatment without adverse drug side effects (103).

While its use as a therapy for macular degeneration is now secure, the anti-angiogenic properties of anti-VEGF aptamers could be applied to a variety of diseases. Based on previous findings (104, 105), Grover *et al.* showed that hypertension downregulates VEGF expression in lung tissue leading to symptoms consistent with PPHN (persistent pulmonary hypertension of the newborn). In order to monitor changes in VEGF expression in lung tissue as a result of hemodynamic stress, previous models inducing hypertension used surgical methods to physically constrict the arteries. In contrast, EYE001 was administered to pregnant ewes via catheter to cardiopulmonary arteries to induce chronic VEGF inhibition (106) and thus to better elucidate the role of VEGF role in intrauterine hypertension. Arguably, physical manipulation to achieve hypertension is more invasive than aptamer delivery through catheter placement.

6.1.2. Other physical methods

Additional methods of delivery range from physical manipulation of individual cells to surgical

implantation in a tissue. Aptamers can be directly introduced into cells by microinjection; aptamer-treated cells with particular phenotypes could subsequently be expanded or even xenografted into an organism. In one example, an anti-E2F aptamer was delivered to fibroblasts *in vitro* (107) and inhibition of cell proliferation was observed. Microinjection was a particularly good delivery technique for this particular aptamer because of its relatively high K_d (10^{-7} M); it is likely that high doses of aptamer would have been required to elicit a similar effect if it was passively delivered.

Introduction or implantation of drug delivery devices is a field unto itself, and it is unsurprising that aptamers can be similarly administered using these technologies. In a particularly innovative physical method exploiting microspheres, an anti-VEGF aptamer was released to cornea tissue *in vivo*. Poly(lactic-*co*-glycolic)acid (PLGA) microspheres containing anti-VEGF RNA aptamer (EYE001) were non-invasively placed on harvested rabbit sclera and were shown to release aptamer in a controlled and sustainable manner. To confirm the bioactivity of the released aptamer, proliferation of VEGF-induced HUVECs (human umbilical vein endothelial cells) was monitored. The aptamer-PLGA microspheres demonstrated pronounced inhibition of proliferation over 20 days (108). While these studies were performed *in vitro*, this system aspires to replace the current regimen of intravitreal injections being used in clinical trials (103).

Another eye tissue study employed aptamers for inhibition of bFGF-mediated angiogenesis in the cornea. Briefly, after 11 rounds of selection an anti-angiopoietin-2 (Ang2) aptamer formed a complex with a K_d of 3.1 nM, but bound to the related Tie2 agonist, Ang1, with a K_d >1 μM. The 2'-fluoro modified RNA Ang2 aptamer was shown to inhibit neovascularization when surgically implanted into live rat cornea. "Slow-release pellets" were used to deliver the aptamer in a controlled fashion. The lyophilized pellets consisted of bFGF, sucrose octasulfate aluminum complex (sulcrafate), the modified RNA aptamer, and Hydron polymer. Surgical creation of superficial corneal micropockets provided an environment for aptamer complex to diffuse into surrounding tissue (109). In the aptamer-treated group, up to 40% reduction in neovascular development was observed. These studies have helped to clarify the role of Ang2 *in vivo*, and have also identified an additional target for anti-angiogenesis therapies.

Finally, it is even possible that aptamers could be delivered orally. The *in vivo* stability of an orally administered "hybrid oligo" 25mer was examined. The ^{35}S-labeled phosphorothioate oligo with stabilizing 2'-O-methyl-oligoribonucleotides present at both 3' and 5' ends was orally delivered at a dose of 50 mg/kg to rats and was found to be present in the gut up to 6 hours later. Absorbed through the GI tract, intact oligo was detected in plasma and various tissues, while degradation products predominated in urine (110).

6.1.3. Targeting/ localization

While numerous methods have been used to successfully deliver aptamers into cells and organisms, the therapeutic utility of aptamers would also be enhanced by being able to localize aptamers to a specific site of action. Dougan *et al.* have shown that biodistribution and clearance of biotin-strepdavidin-conjugated anti-thrombin aptamers from the blood was not limited to the expected targets, the liver and kidneys. In fact, 85-92% of the aptamers were distributed to other organs. Almost 50% of the aptamers were found in the liver, bladder, lung, and heart (111). This finding bodes well for the potential delivery of aptamer to a number of different organs and tissues.

Targeting a specific subset of cells based on tissue type, expression profile, or infected or transformed state should allow not only aptamers but other nucleic acid based therapeutics to reach their full potential. Many surface antigens and receptors are potential cell-specific targets (reviewed in (112)) and have been exploited with some success. Epidermal growth factor receptor (EGF-R) is highly expressed in lung cancer tissue. Recombinant EGF incorporated into a polylysine/DNA transfection complex effectively directed DNA to lung cancer cells, but not to colon carcinoma cells (113). Similarly, the folate receptor is overexpressed in many tumor cells. Upon binding, the receptor is internalized through a non-endocytic process, protolysis. This internalization could potentially be exploited for delivery of agents directly into cells (114, 115). In one demonstration of this, Li *et al.* conjugated c-fos antisense oligonucleotides to folate. Uptake of the conjugates into ovarian cancer cells was increased 8-fold compared to control cells (116). The prostate-specific membrane antigen (PSMA) is considered an excellent prostate tumor cell marker. PSMA is primarily overexpressed in prostate tumor cells and is seen at very low levels in brain, salivary glands, and gut (117). As previously discussed, this marker has been targeted by *in vitro* selection. Using the previously developed xPSM-A10 anti-PSMA aptamer (59), Farokhzad et. al showed that nanoparticle bioconjugates could specifically bind and be taken up into prostate cancer cells *in vitro* (60). The aptamer served as an escort to prostate cell membrane and the nanoparticle polymer served as a controlled release mechanism for a model drug.

Similarly, anti-hNE aptamers served as a guide for an otherwise weak but covalent hNE inhibitor, valyl phosphonate. A random sequence pool was annealed to splint DNA conjugated to the inhibitor, and aptamers that could direct the covalent modification of hNE were selected (118, 119). The aptamer directs the otherwise relatively poor organic irreversible inhibitor, valyl phosphonate, to the enzyme active site with a second order inhibition rate constant of 10-30 $nM^{-1}min^{-1}$, significantly higher than either the aptamer or the organic inhibitor alone (101, 118).

These so-called escort aptamers are just emerging as potential therapetics. Escort aptamers can be conjugated to radiolabels, drugs or other cytotoxic agents, enhancing delivery, targeting, or other pharmacokinetic properties. Thus far, escort aptamers have primarily been used for *in vivo* imaging and diagnostics (reviewed in (120)). For

example, Charlton *et al.* used fluorescently labeled anti-hNE aptamers to image activated neutrophils at sites of inflammation in rats. A peak signal to background ratio was achieved 2 hours after injection whereas IgG controls took 3 hours(118). Hilger *et al.* have conjugated the isotope Tc-99m to anti-L-selectin RNA, potentiating radiolabel delivery for therapy and imaging (121).

In general, only antibodies have rivaled aptamers for specificity of targeting. Aptamers, though, have a number of advantages over antibodies for escorting and *in vivo* imaging (reviewed in (120)). Because of their small sizes, aptamers can penetrate into tissues and cells more effectively than antibodies. This attribute coupled with an aptamer's faster clearance rate allows for quick targeting and rapid removal of potentially cytotoxic agents. For example, therapeutic radiolabels should work best when they can be quickly removed from non-target tissues. The faster clearance rate of aptamers also contributes to a higher target:background ratio of signal. Hicke and Stephens showed that an anti-tumor aptamer achieved a high tumor:blood distribution ratio in mice within hours, whereas an antibody to the same protein took days to achieve significant tumor:blood ratios. Even then, the antibody tumor:blood ratio was notably lower than that of the aptamer. In spite of the faster clearance of aptamer, molar uptake of aptamer was only 2-fold less than an equivalent dose of antibody (120). Because of their relatively low toxicity and faster clearance, multiple dosages of escort aptamers can potentially be applied to compensate for this lowered uptake. In situations where a longer circulating aptamer duration is required for more complete labeling, aptamers can be modified to extend their bloodstream half-life (see Section 5.1.4).

6.1.4. Aptamer stabilization and pharmacokinetics

Unmodified, RNA and DNA can begin degrading in serum within minutes, and a number of nucleic acid modifications have been introduced over the years to stabilize nucleic acids for *in vivo* applications, including the use of modified bases, phosphorothioate linkages, peptide backbones, DNA/RNA chimeras, and Spiegelmers (reviewed in (2, 122, 123)). In one example, Jellinek *et al.* showed that 2'-aminopyrimidine-modified RNA can remain relatively stable for days (124). Similarly, Pieken *et al.* showed that incorporating 2'-fluoropyrimidine or 2' aminopyrimidine modifications into a hammerhead ribozyme increased its stability in serum greater than 1000-fold (125). Modifications can also be appended to the ends of aptamers to confer greater stability. A 3'-3' linkage, for example, can be added to prevent 3' exonuclease degradation (reviewed in (122)).

Modified residues can be introduced during either enzymatic or chemical synthesis, or can be added post-transcriptionally. Commercial transcription kits are now available that can incorporate modified bases into a transcript making the production of stabilized aptamers much more practical. Modified polymerases that can accommodate the modified residues are also beginning to be produced, and a selection scheme was recently reported that yielded a T7 polymerase capable of incorporating three of the four 2'-O-methyl NTPs (126).

While chemical modifications lead to aptamers that are not as readily degraded, this does not solve the problem of physiological clearance from the blood. Lee *et al.* found the average initial half-life of a DNA 15-mer introduced into cynomolgus monkeys by constant infusion (at 0.1, 0.3 or 0.5 mg/kg/min) or bolus injection (at 11.25 or 22.5 mg/kg) was just 1.4 minutes (127). Several groups have found that the rate of nucleic acid clearance could be reduced by generating high molecular weight conjugates. Tucker *et al.* and others have attached 40 kD polyethylene glycol (PEG) moieties to the ends of the anti-VEGF aptamer, EYE001, and subsequently extended aptamer half-life to 9.3 h after i.v. administration (128, 129). Dougan *et al.* attached biotin to the 3' ends of anti-thrombin aptamers and showed a reduction in degradation. However, clearance from rabbit and mouse bloodstreams was not affected. Further appending strepdavidin to the aptamers' termini extended the lifetime of the biotinylated anti-thrombin aptamers by 10 to 20-fold (111). Attaching lipids to aptamer ends has also been shown to extend plasma lifetime (130, 131). In the case of controlled release of an anti-VEGF aptamer from PLGA microspheres (see Section 5.1.2 above), it was determined that initial co-lyophilization of the aptamer with trehalose before encapsulation allowed slow and steady release of the aptamer (2 microgram/day) over the course of 20 days *in vitro*. The released aptamers were able to inhibit proliferation of cultured human umbilical vein endothelial cells. PLGA-aptamer conjugates were delivered directly into rabbit sclera, and their timed release over 6 days was shown to be comparable to that observed in the *in vitro* studies (108).

6.1.5. Controlling aptamer dosage

As aptamers move further toward use *in vivo*, being able to control the 'dosage' of aptamer therapies may prove useful. Even though aptamers have so far shown little or no toxicity, it may nonetheless be useful to shut off or modulate the effects of an aptamer.

Aptamer levels in the blood may be controlled simply by further understanding clearance and distribution parameters and modulating aptamer dosage appropriately. Tucker *et al.* administered 1 mg/kg of a stabilized anti-VEGF aptamer into rhesus monkey either subcutaneously (s.c.) or intravenously (i.v.). At time zero, plasma concentration was 25.5 microgram/mL in i.v.-treated animals and only about 0.1 microgram/mL in animals receiving aptamers s.c.. Eight to twelve hours after administration, though, animals in both treatment groups had reached a plasma concentration of approximately 5 microgram/mL. Elimination half-life was 9.3 hrs for i.v.-administrated aptamers and 12 hrs for s.c.-administered aptamers (128). Initial high plasma concentrations can therefore be best achieved by i.v. administration. However, aptamers showed slightly longer duration of circulation with s.c. administration.

In one strategy where aptamers were used as anticoagulants, the Sullenger group at Duke University selected aptamers that bound to factor IXa and then developed an antisense 'antidote.' Inhibition of factor IXa prevents activation of thrombin thereby stopping the blood

Figure 2. Advantages and disadvantages of some viral delivery vectors.

clotting pathway. Human plasma was treated with aptamers, then with 'antidote' oligonucleotides complementary to the aptamer sequence. Reversal of the aptamer anti-clotting effect was seen within 10 minutes (132). This response time is comparable to the protamine treatment typically used to reverse the effects of the more commonly used anticoagulant, heparin. Protamine, though, has been known to trigger immune responses in some patients, and an overdose of protamine can actually lead to more excessive bleeding (reviewed in (133)).

More recently, Rusconi *et al.* demonstrated the efficacy of their antidotes *in vivo*. A cholesterol moiety was appended to the anti-factor IXa aptamer (Ch-9.3t) and increased the aptamer's half-life in the porcine bloodstream to 1-1.5 hours, relative to 5-10 minutes for the unconjugated aptamer. The cholesterol modified aptamer had anticoagulant properties similar to the uconjugated aptamer, and increased the activated clot time by 73.5 s. A bolus injection (5 mg/kg) of an antidote oligonucleotide, 5-2C, then reduced the anticoagulant effects of the aptamer by 95% over 10 min. Interestingly, while the half-life of the antidote alone was comparable to that of other 2'-O-methyl oligonucleotides (on the order of minutes), when associated with the aptamer in the blood, anti-clotting effects could be observed for over an hour (134).

6.2. Endogenous delivery

While aptamers can be introduced locally or systemically to antagonize the function of extracellular targets, endogenous delivery or gene therapy approaches may prove useful for modulating the activities of intracellular targets. So-called 'intramer' therapies would involve continuous expression of a therapeutic aptamer within a cell. However, efficiently delivering aptamers into cells remains a prominent challenge. Research has so far focused on developing vectors or vehicles that can efficiently enter cells, express high levels of aptamers, and remain non-toxic.

6.2.1. Cellular transduction

To advance intramer or gene therapies, a number of different strategies have been pioneered to deliver aptamer expression cassettes directly into cells. Amongst the most popular have been viral vectors, which have the advantage of being both delivery mechanisms and expression cassettes. Over the years, viral vectors have been radically modified to better regulate expression levels, localize transcripts, and transfect different types of cells. The different types, advantages, disadvantages, and uses of viral vectors (Figure 2) have been extensively discussed elsewhere in the literature, and therefore will not be analyzed here (reviewed in (135, 136)). Suffice it to say that viral vectors have remained the most efficient and commonly used means of delivering nucleic acids *in corpus*. However, safety issues remain a significant concern. Retroviruses, for example, could potentially integrate into undesirable locations in the host genome causing insertion mutations. They could also recombine with naturally occurring or endogenous retroviruses to regain function, or worse, give rise to a novel virus.

Different approaches have been applied to reduce safety concerns, including the removal of key genes involved in pathogenicity. For example, retroviruses generally consist of three major structural genes (*gag*, *pol* and *env*) flanked by 5' and 3' LTRs. These genes give rise to proteins necessary for infection, reverse transcription, integration, and packaging. In the simplest class of retroviral vectors, therapeutic constructs are cloned into a provirus in which all the genes involved in packaging and budding have been removed from the viral genome. The provirus is transfected into a packaging cell line which provides all the necessary proteins in *trans* to make complete viruses. Viruses can thereby be generated that can only infect and integrate once. An intramer construct could potentially be expressed from the viral LTR or internally cloned promoters.

Viral delivery was used by Bai *et al.* to test the safety of anti-HIV nucleic acids. These authors cloned an anti-HIV aptamer and anti-HIV ribozymes into a Gag- and Pol-deficient Moloney murine leukemia virus (MMLV) backbone. The vector was transfected into 293T packaging cells stably expressing Gag and Pol genes, and product virus was used to transduce human CD34(+) cells.

Expression of the small nucleic acids did not affect CD34(+) growth and differentiation in SCID-hu mice. Moreover, cells transduced with the antiviral constructs showed marked resistance to the early stages of HIV infection (12 days). Later timepoints still showed some resistance, although it was not as pronounced. Interestingly, only a small fraction of the treated cells, likely a dividing sub-population, were stably transduced but were nonetheless able to expand and confer long-term prophylactic effects. By using a lentiviral vector which could infect non-dividing cells, even greater transfection efficiencies and anti-viral efficacies might be achieved (23).

6.2.2. Cellular transfection

Although efforts have been made to inactivate viral vectors, and next generation vectors are likely significantly safer, there remains the fear that any viral preparation that will be used for gene delivery may still contain replication competent virus. Plasmids eliminate the safety concerns associated with viruses and have somewhat greater flexibility of design. However, finding an efficient means to deliver plasmids has been a challenge. Cells, particularly muscle and skin, have been shown to readily take up nucleic acids delivered in their proximity, but the mechanism is unknown and gene expression is typically short-lived.

The most popular vehicles for the delivery of plasmid or other expression vectors are lipid amalgams, particularly cationic lipids (reviewed in (137-139)). The lipids interact to form liposomes, and the positively charged head group can hold tightly to negatively charged nucleic acids. Following endocytosis, the nucleic acids can be released within the host cell.

Liposomes offer a quick and efficient means of delivering genes into tissue culture cells and *ex vivo* systems but have not demonstrated the same success *in corpus*. Liposomal delivery into animals and humans has been relatively inefficient, unstable and, in some cases, cytotoxic compared to viral-mediated delivery. Studies have shown that liposomes are rapidly cleared, readily disrupted under physiological conditions, or otherwise neutralized by endogenous factors (reviewed in (139-141)).

Even so, lipids have been used for gene delivery in clinical trials (142-144) and have shown great promise when optimized formulations are used or when combined with other delivery techniques. Adding PEG moieties, for example, has helped to stabilize liposomal complexes (lipoplexes), both in terms of resistance to inactivation and longer circulation times. For example, methoxypolyethylene glycol-conjugated liposomes circulate on the order of hours, compared to minutes for non-conjugated liposomes (reviewed in (145)). Nicolazzi *et al.* investigated differently charged PEG-lipid formulations and showed that a bi-anionic PEG-lipid complex increased circulating lipoplex concentrations by 5- to 7-fold 30 min after injection into mice (146). Ultimately, by using different sized PEGs and altering the charge or chemistry of the PEGylated complexes it has proven possible to craft 'stealth liposomes' that stabilize the complex, prevent neutralizing interactions, and evade immune responses (reviewed in (145)). However, the length and charge of PEGylated liposomal complexes can affect transfection efficiency, and thus these affects must be balanced in any given formulation (reviewed in (147-149)).

The chemistry surrounding PEG moieties is exceptionally versatile. PEG conjugates can be prepared which are chemically cleavable or biodegradable, and can therefore better release genes after entry into the cell. Targeting sequences or signals can be readily appended to PEG, and the resultant nucleic acid complexes have been used to target expression vectors to tumor endothelium cells and lung cells (reviewed in (140, 150)).

Lipoplexes have also been modified with peptides to confer stability, enhanced uptake into cells or nuclear localization of genes (reviewed in (151)). Attachment of the plasma iron transporter, transferrin, to lipoplexes has been shown to increase endocytosis of liposomes and generally increased transfection efficiency. Similarly, treating lipoplexes with poly-lysine or protamine sulfate can also increase transfection into cells. It has been proposed that such cationic peptides act to condense nucleic acids, making the lipoplex tighter, more stable, and ultimately easier to transport. The combination of transferrin and protamine has been shown to act synergistically in further increasing transfection efficiencies (152, 153).

6.2.3. Other delivery methods

Aside from lipids and viral vectors, other biopolymers are also being exploited for gene delivery. Polyethylenimines (PEIs) have promoted efficient transfection into cells (reviewed in (154-156)). Gelatin, chitosan and other compounds have been also been used to make microspheres that can transfer nucleic acids (reviewed in (137, 157)). One novel and promising technique that has emerged in recent years involves using nanoparticles for delivery. Nanoparticles are submicron materials or capsules that can be made from a variety of synthetic polymers. Because of their small size, nanoparticles readily access deep tissue and are taken up into cells in a time- and concentration-dependent manner. Nanoparticles have shown remarkable stability in serum and are easily modified. For example, nanoparticles have been made that are essentially biodegradable, allowing for time-release delivery of therapeutics (reviewed in (158-160)). Anti-PSMA aptamers have been conjugated to nanoparticles and effectively delivered into prostate tumor cells (see Section 3.2.1).

Inorganic nanoparticles, such as quantum dots (QDs) may also prove useful for both delivery and diagnostics. QDs are fluorophores which have the ability to absorb across a broad range of wavelengths while emitting within a very discreet range. QDs, like their other nanoparticle cousins, can be readily taken up into cells and have been employed for *in vivo* imaging. Modifications of the surface have also allowed for conjugation to various compounds and increased circulation stability. Ballou *et al.*

found that QDs stabilized with PEG and delivered into mice could still be seen after 1 month in the liver, lymph nodes and bone marrow. After four months, fluorescence was just barely detectable, but QDs could still be seen in the lymph nodes and bone marrow (161). QD conjugates may provide the ability to track the delivery of therapeutics in real time. For example, Gao *et al.* were able to image the localization of anti-PSMA antibody conjugates in live mice using a whole-body illumination system (162).

Techniques as well as materials can be varied in order to enhance delivery. For example, liposomes do not appear to promote nucleic acid delivery into muscle tissue. Instead, Mir *et al.* injected a luciferase expressing plasmid into the leg muscle of mice and electric pulses were applied (8 pulses at 20 ms per pulse, 200 V/cm; 1 Hz) and a reproducible and significant 10 to 100-fold increase in luciferase expression was detected over the course of the experiment (ranging from a 25-fold increase over non-electrotransferred control at 3 hrs to a 734-fold increase on day 121). Similar experiments in other animals (rat, rabbit, monkey) with other reporters yielded reproducible but more modest effects (163). Electroporation has also been used in several clinical trials and has proven particularly well-suited for targeting tumors and melanomas (reviewed in (164)). Recently, electroporation has been used for the high efficiency transfection of a number of previously difficult-to-transfect primary cells such as CD34+ cells, lymphocytes, cardiomyocytes and neurons by electrotransferring DNA directly into the nucleus. This technique, called 'nucleofection', also has the added benefits of delivering into cells without the need for mitotic division and allowing for expression of a transgene in hours rather than days ((165)). Because of this, electroporation may prove important for *ex vivo* transfection of progenitor cells for stem cell therapy.

Another device, the gene gun, makes use of high pressure to deliver DNA-coated gold particles directly into cells. While ballistic transfection has been successful, unintended damage to cells has been an issue and delivery with the gene gun has so far been limited to a short subcutaneous depth range. Newer designs in gene gun technology, though, have significantly reduced cell damage, and DNA can now be biolistically delivered to animal liver (166) and heart (167).

6.2.4. Expression cassettes

To achieve continuous and high intracellular expression levels from introduced vectors, many different promoter systems and expression cassettes have been designed. Most cassettes employ a strong promoter; both Pol II and Pol III promoters have both been used extensively for aptamer expression. Pol II transcripts generally consist of mRNAs and snRNAs, and tend to be relatively short-lived, but are more actively transcribed. Conversely, Pol III transcripts, such as tRNAs, U6, and 5S RNA, are not transcribed as frequently, but are very stable (reviewed in (168, 169)). Some promoters are cell-type specific, providing another option for aptamer targeting. For example, the prostate specific antigen promoter (PSAP) has been shown to drive expression in prostate cell lines, but not in non-prostate or PSA- cell lines (170). Similarly, tissue-specific promoters have been identified for several cell types including, but not limited to: bone (171, 172), liver (173, 174), endothelial (175, 176) skin melanoma (177) and brain glioma cells (178). Expression constructs also incorporate various processing signals to aid in aptamer localization or stability. Levels or sites of expression can be manipulated by including enhancers, nuclear localization signals (NLSs) or locus control regions (LCRs). For example, a minimized PSMA enhancer and PSMA promoter were used to drive the expression of cytosine deaminase, which converts the non-toxic prodrug 5-fluorocytosine to toxic 5-fluorouracil. Prostate tumor cells, C4-2, were transfected with the construct and treated with the prodrug. Cells expressing the deaminase showed greater than 50-fold decrease in prodrug IC_{50} illustrating the suicidal effect of the expressed construct. In contrast, lung, colon and breast cell lines did not show significant change in cell toxicity (179).

Because of the typically small size of aptamers and the necessity for aptamers to fold properly to be functional, it has become common to include processing signals or ribozymes that will cleave an aptamer from a nascent RNA transcript. In an early example of this, Sullenger *et al.* designed a vector that expressed small RNAs as a tRNA chimera. Processing signals were placed at the 3' end of the chimera to ensure transcription termination (180). Later modifications included a processing signal upstream of the aptamer cloning site so that aptamers could be cleaved from the tRNA chimera and a 3' hairpin structure to stabilize the RNA (181). The chimera was expressed from a $tRNA_{met}^{i}$ promoter cloned into the 3'LTR of an MMLV vector backbone, and recombinant proviral DNA was transfected into packaging cell lines to grow viral particles. By putting the expression cassette into the 3' LTR, transduced recombinant retroviruses would be expressed twice, from both the integrated 5' UTR and 3' LTR (182).

These 'double-copy' vectors have now been used numerous times to demonstrate the inhibition of targets by small, expressed nucleic acids in transduced tissue culture cells (183-185). However, using an earlier version of the vector Martell *et al.* found that a previously selected aptamer against transcription factor E2F (107) had low binding efficiency (186). The aptamer formed complexes with a K_d of 2-4 nM *in vitro*, but when expressed in the context of the vector sequences the K_d increased to 200 nM. Martell *et al.* surmised that flanking sequences from the expression construct impaired folding of the aptamer and attempted to repair these defects via a so-called expression cassette selection. Random sequence regions were added to either side of the aptamer and *in vitro* selections were performed with the expression cassette RNA pool. Re-selected aptamers had a K_d of 15 nM and could now be effectively expressed in cells.

More recent expression constructs for the *in vivo* expression of aptamers have made use of cis-acting ribozymes to release aptamers. Joshi and Prasad flanked anti-RT aptamer sequences with hammerhead ribozymes

behind a CMV promoter and showed strong inhibition of HIV replication in tissue culture cells (19). Nishikawa *et al.* effectively expressed anti-HCV aptamers by lodging them between HDV ribozymes (see Section 3.1.1) (34).

6.2.5. Regulating gene expression.

So far we have primarily considered aptamers as therapeutics that modify the activities of proteins. In addition, though, it may be possible to use aptamers as control elements for gene expression. Aptamer-mediated gene expression may prove useful in designing gene therapies. Just as an 'antidote' controls the activity of an exogenous aptamer, a small organic could control the activity of a therapeutic protein. To this end, Werstuck and Green introduced aptamers against antibiotics (Kan A and tobramycin) or Hoechst dyes into the 5' untranslated region (UTR) of a reporter gene, in the hopes that drug- or dye-binding would modulate transcription or translation. After proofing their system *in vitro*, Werstuck and Green cloned two anti-dye aptamers, H10 and H19, into the 5' UTR of beta-galactosidase and assayed enzyme activity in the presence and absence of Hoescht dye H33342. Expression was mediated in a dye-dependent manner with up to 90% reduction in beta-galactosidase expression at 10 mM Hoescht dye (187). Likewise, Suess *et al.* inserted an anti-tetracycline (tet) aptamer near the start codon of GFP in yeast cells and found tetracycline-mediated inhibition of translation (188). Regulatory aptamers may have even more general utility for the control of cell biology. Grate and Wilson introduced a malachite green binding motif upstream of CLB2, one of the cyclins that directs transition from G2 to mitosis. Cell cycle progression was disrupted in yeast in the presence of a malachite green analog (189).

Modulating the conformation of a 5' UTR with a small organic was a strategy that was apparently chanced upon by Nature long before it was envisioned by biotechnology. In 2002, Ron Breaker and his co-workers characterized the first so-called riboswitch, a regulatory RNA sequence found upstream of thiamine biosynthetic genes. The thiamine riboswitch could bind thiamine, undergo a conformational change, and create an RNA structure that effectively blocked translation of the downstream gene (190). A number of additional riboswitches have now been found and have been shown to regulate transcription termination as well as translation initiation (reviewed in (191-194)).

Mirroring the naturally found riboswitches, transcriptional activators and translational inhibitors have been designed based on aptamer affinity. Buskirk *et al.* attached random pool RNA to MS2 hairpins that directed the sequences to an MS2 coat protein fused to LexA in a yeast system. Interaction of functional RNAs with the LexA operator transcription unit promoted the production of His3 which allowed for survival in histidine-deficient media. One clone activated transcription 53-fold greater than the common transcriptional activator, Gal4 (195). Mandal *et al.* cleverly combined selection technology with rational design to produce aptamer-based translational inhibitors. Stem loops placed near the ribosome binding site (RBS) upstream of a beta-gal reporter gene were shown to inhibit translation. An anti-theophylline aptamer was attached to a previously selected communication module (196) and replaced the stem-loop. Upon binding of theophylline, the aptamer-construct shifted the regulatory unit by one nucleotide and recovered translational activity (197).

While effector-dependent regulation of gene expression may allow the construction of intricate synthetic genetic circuits, aptamers can also potentially regulate proteins in an effector-dependent manner. Vuyisich and Beal employed a novel selection scheme to produce anti-protein aptamers that would be activated by neomycin (198). In this selection, 6 rounds of selection were performed using standard selection procedures against the DNA repair enzyme formamidopyrimidine glycosylase (Fpg). Then further rounds were carried out in which bound RNAs were eluted in the presence of neomycin. Selected aptamers were able to bind Fpg in the low nanomolar range (7.5 nM) and could inhibit 1 nM Fpg at 100 nM. In addition, though, the aptamers now lost their ability to inhibit Fpg activity with increasing neomycin concentrations; this property was not observed in a control selection that did not employ neomycin elution. The authors envisioned that such a system could be readily adapted as an on-off switch for pathway and cell cycle studies.

7. PERSPECTIVES

While *in vitro* selection of aptamers has been an established technology for over a decade, its utility has principally been for the study of proteins and mechanisms *in vitro*. The use of aptamers as drugs has been forestalled in part by issues relating to delivery, stability, and pharmacokinetics. However, recent progress on these issues has paved the way to the use of aptamers in clinical diagnostics and therapeutic applications. Importantly, the first clinical studies that involve aptamers have been carried out, and Macugen (EYE001) has been approved by the FDA. Additional, medically relevant targets have yielded aptamers with high affinities and specificities , a number of approaches have been validated for stabilizing and delivering aptamers *in corpus*, and animal and preclinical experimentation with aptamers are bringing along the next drugs in the pipeline. As gene therapy becomes an increasingly well-established technology, the use of intracellular aptamers (intramers) may prove to be a unique and extremely viable strategy for disrupting pathological signal transduction or other metabolic or regulatory pathways.

8. ACKNOWLEDGEMENT

This work was supported by NIH grant AI36083.

9. REFERENCES

1. Ellington, A. D. & R. Conrad: Aptamers as potential nucleic acid pharmaceuticals. *Biotechnol Annu Rev*, 1, 185-214 (1995)

2. Famulok, M. & G. Mayer: Aptamers as tools in molecular biology and immunology. *Curr Top Microbiol Immunol*, 243, 123-36 (1999)
3. Marshall, K. A. & A. D. Ellington: *In vitro* selection of RNA aptamers. *Methods Enzymol*, 318, 193-214 (2000)
4. Jenison, R. D., S. C. Gill, A. Pardi & B. Polisky: High-resolution molecular discrimination by RNA. *Science*, 263, 1425-9 (1994)
5. Conrad, R., L. M. Keranen, A. D. Ellington & A. C. Newton: Isozyme-specific inhibition of protein kinase C by RNA aptamers. *J Biol Chem*, 269, 32051-4 (1994)
6. Kensch, O., B. A. Connolly, H. J. Steinhoff, A. McGregor, R. S. Goody & T. Restle: HIV-1 reverse transcriptase-pseudoknot RNA aptamer interaction has a binding affinity in the low picomolar range coupled with high specificity. *J Biol Chem*, 275, 18271-8 (2000)
7. Tuerk, C., S. MacDougal & L. Gold: RNA pseudoknots that inhibit human immunodeficiency virus type 1 reverse transcriptase. *Proc Natl Acad Sci U S A*, 89, 6988-92 (1992)
8. Homann, M. & H. U. Goringer: Combinatorial selection of high affinity RNA ligands to live African trypanosomes. *Nucleic Acids Res*, 27, 2006-14 (1999)
9. White, R., C. Rusconi, E. Scardino, A. Wolberg, J. Lawson, M. Hoffman & B. Sullenger: Generation of species cross-reactive aptamers using "toggle" SELEX. *Mol Ther*, 4, 567-73 (2001)
10. Seiwert, S. D., T. Stines Nahreini, S. Aigner, N. G. Ahn & O. C. Uhlenbeck: RNA aptamers as pathway-specific MAP kinase inhibitors. *Chem Biol*, 7, 833-43 (2000)
11. Famulok, M., M. Blind & G. Mayer: Intramers as promising new tools in functional proteomics. *Chem Biol*, 8, 931-9 (2001)
12. Famulok, M., G. Mayer & M. Blind: Nucleic acid aptamers-from selection *in vitro* to applications *in vivo*. *Acc Chem Res*, 33, 591-9 (2000)
13. Kim, D. H., M. Longo, Y. Han, P. Lundberg, E. Cantin & J. J. Rossi: Interferon induction by siRNAs and ssRNAs synthesized by phage polymerase. *Nat Biotechnol*, 22, 321-5 (2004)
14. Malmegrim, K. C., G. J. Pruijn & W. J. van Venrooij: The fate of the U1 snRNP autoantigen during apoptosis: implications for systemic autoimmunity. *Isr Med Assoc J*, 4, 706-12 (2002)
15. Burlingame, R. W. & R. L. Rubin: Autoantibody to the nucleosome subunit (H2A-H2B)-DNA is an early and ubiquitous feature of lupus-like conditions. *Mol Biol Rep*, 23, 159-66 (1996)
16. Van Bruggen, M. C., C. Kramers & J. H. Berden: Autoimmunity against nucleosomes and lupus nephritis. *Ann Med Interne (Paris)*, 147, 485-9 (1996)
17. Kohn, D. B., G. Bauer, C. R. Rice, J. C. Rothschild, D. A. Carbonaro, P. Valdez, Q. Hao, C. Zhou, I. Bahner, K. Kearns, K. Brody, S. Fox, E. Haden, K. Wilson, C. Salata, C. Dolan, C. Wetter, E. Aguilar-Cordova & J. Church: A clinical trial of retroviral-mediated transfer of a rev-responsive element decoy gene into CD34 (+) cells from the bone marrow of human immunodeficiency virus-1-infected children. *Blood*, 94, 368-71 (1999)
18. Bukh, J., R. H. Miller & R. H. Purcell: Genetic heterogeneity of hepatitis C virus: quasispecies and genotypes. *Semin Liver Dis*, 15, 41-63 (1995)
19. Joshi, P. & V. R. Prasad: Potent inhibition of human immunodeficiency virus type 1 replication by template analog reverse transcriptase inhibitors derived by SELEX (systematic evolution of ligands by exponential enrichment). *J Virol*, 76, 6545-57 (2002)
20. Good, P. D., A. J. Krikos, S. X. Li, E. Bertrand, N. S. Lee, L. Giver, A. Ellington, J. A. Zaia, J. J. Rossi & D. R. Engelke: Expression of small, therapeutic RNAs in human cell nuclei. *Gene Ther*, 4, 45-54 (1997)
21. Konopka, K., N. Duzgunes, J. Rossi & N. S. Lee: Receptor ligand-facilitated cationic liposome delivery of anti-HIV-1 Rev-binding aptamer and ribozyme DNAs. *J Drug Target*, 5, 247-59 (1998)
22. Konopka, K., N. S. Lee, J. Rossi & N. Duzgunes: Rev-binding aptamer and CMV promoter act as decoys to inhibit HIV replication. *Gene*, 255, 235-44 (2000)
23. Bai, J., N. Banda, N. S. Lee, J. Rossi & R. Akkina: RNA-based anti-HIV-1 gene therapeutic constructs in SCID-hu mouse model. *Mol Ther*, 6, 770-82 (2002)
24. Fisher, T. S., P. Joshi & V. R. Prasad: Mutations that confer resistance to template-analog inhibitors of human immunodeficiency virus (HIV) type 1 reverse transcriptase lead to severe defects in HIV replication. *J Virol*, 76, 4068-72 (2002)
25. Schneider, D. J., J. Feigon, Z. Hostomsky & L. Gold: High-affinity ssDNA inhibitors of the reverse transcriptase of type 1 human immunodeficiency virus. *Biochemistry*, 34, 9599-610 (1995)
26. Nishikawa, S., F. Nishikawa & K. Fukuda: *In vitro* selection of RNA aptamers against HCV-NS3 helicase and their structural similarity with 3' (+)UTR of HCV. *Nucleic Acids Res Suppl*241-2 (2003)
27. Strader, D. B., T. Wright, D. L. Thomas & L. B. Seeff: Diagnosis, management, and treatment of hepatitis C. *Hepatology*, 39, 1147-71 (2004)
28. Vial, T. & J. Descotes: Clinical toxicity of the interferons. *Drug Saf*, 10, 115-50 (1994)
29. Pavio, N. & M. M. Lai: The hepatitis C virus persistence: how to evade the immune system? *J Biosci*, 28, 287-304 (2003)
30. Fukuda, K., D. Vishinuvardhan, S. Sekiya, N. Kakiuchi, K. Shimotohno, P. K. Kumar & S. Nishikawa: Specific RNA aptamers to NS3 protease domain of hepatitis C virus. *Nucleic Acids Symp Ser*237-8 (1997)
31. Fukuda, K., D. Vishnuvardhan, S. Sekiya, J. Hwang, N. Kakiuchi, K. Taira, K. Shimotohno, P. K. Kumar & S. Nishikawa: Isolation and characterization of RNA aptamers specific for the hepatitis C virus nonstructural protein 3 protease. *Eur J Biochem*, 267, 3685-94 (2000)
32. Kumar, P. K., K. Machida, P. T. Urvil, N. Kakiuchi, D. Vishnuvardhan, K. Shimotohno, K. Taira & S. Nishikawa: Isolation of RNA aptamers specific to the NS3 protein of hepatitis C virus from a pool of completely random RNA. *Virology*, 237, 270-82 (1997)
33. Urvil, P. T., N. Kakiuchi, D. M. Zhou, K. Shimotohno, P. K. Kumar & S. Nishikawa: Selection of RNA aptamers that bind specifically to the NS3 protease of hepatitis C virus. *Eur J Biochem*, 248, 130-8 (1997)
34. Nishikawa, F., N. Kakiuchi, K. Funaji, K. Fukuda, S. Sekiya & S. Nishikawa: Inhibition of HCV NS3 protease by RNA aptamers in cells. *Nucleic Acids Res*, 31, 1935-43 (2003)

35. Fukuda, K., T. Umehara, S. Sekiya, K. Kunio, T. Hasegawa & S. Nishikawa: An RNA ligand inhibits hepatitis C virus NS3 protease and helicase activities. *Biochem Biophys Res Commun*, 325, 670-675 (2004)
36. Kanai, A., K. Tanabe & M. Kohara: Poly (U) binding activity of hepatitis C virus NS3 protein, a putative RNA helicase. *FEBS Lett*, 376, 221-4 (1995)
37. Aldaz-Carroll, L., B. Tallet, E. Dausse, L. Yurchenko & J. J. Toulme: Apical loop-internal loop interactions: a new RNA-RNA recognition motif identified through *in vitro* selection against RNA hairpins of the hepatitis C virus mRNA. *Biochemistry*, 41, 5883-93 (2002)
38. Kikuchi, K., T. Umehara, K. Fukuda, J. Hwang, A. Kuno, T. Hasegawa & S. Nishikawa: RNA aptamers targeted to domain II of hepatitis C virus IRES that bind to its apical loop region. *J Biochem (Tokyo)*, 133, 263-70 (2003)
39. Hilleman, M. R.: Realities and enigmas of human viral influenza: pathogenesis, epidemiology and control. *Vaccine*, 20, 3068-87 (2002)
40. Jeon, S. H., T. Ben-Yedidia & R. Arnon: Intranasal immunization with synthetic recombinant vaccine containing multiple epitopes of influenza virus. *Vaccine*, 20, 2772-80 (2002)
41. Jeon, S. H., B. Kayhan, T. Ben-Yedidia & R. Arnon: A DNA aptamer prevents influenza infection by blocking the receptor binding region of the viral hemagglutinin. *J Biol Chem*, 279, 48410-9 (2004)
42. Pan, W., R. C. Craven, Q. Qiu, C. B. Wilson, J. W. Wills, S. Golovine & J. F. Wang: Isolation of virus-neutralizing RNAs from a large pool of random sequences. *Proc Natl Acad Sci U S A*, 92, 11509-13 (1995)
43. Wang, J., H. Jiang & F. Liu: *In vitro* selection of novel RNA ligands that bind human cytomegalovirus and block viral infection. *Rna*, 6, 571-83 (2000)
44. Goringer, H. U., M. Homann & M. Lorger: *In vitro* selection of high-affinity nucleic acid ligands to parasite target molecules. *Int J Parasitol*, 33, 1309-17 (2003)
45. Kaiser, A., A. Gottwald, C. Wiersch, W. Maier & H. M. Seitz: The necessity to develop drugs against parasitic diseases. *Pharmazie*, 57, 723-8 (2002)
46. Homann, M. & H. U. Goringer: Uptake and intracellular transport of RNA aptamers in African trypanosomes suggest therapeutic "piggy-back" approach. *Bioorg Med Chem*, 9, 2571-80 (2001)
47. Lorger, M., M. Engstler, M. Homann & H. U. Goringer: Targeting the variable surface of African trypanosomes with variant surface glycoprotein-specific, serum-stable RNA aptamers. *Eukaryot Cell*, 2, 84-94 (2003)
48. Ulrich, H., M. H. Magdesian, M. J. Alves & W. Colli: *In vitro* selection of RNA aptamers that bind to cell adhesion receptors of Trypanosoma cruzi and inhibit cell invasion. *J Biol Chem*, 277, 20756-62 (2002)
49. Jellinek, D., L. S. Green, C. Bell & N. Janjic: Inhibition of receptor binding by high-affinity RNA ligands to vascular endothelial growth factor. *Biochemistry*, 33, 10450-6 (1994)
50. Ruckman, J., L. S. Green, J. Beeson, S. Waugh, W. L. Gillette, D. D. Henninger, L. Claesson-Welsh & N. Janjic: 2'-Fluoropyrimidine RNA-based aptamers to the 165-amino acid form of vascular endothelial growth factor (VEGF165). Inhibition of receptor binding and VEGF-induced vascular permeability through interactions requiring the exon 7-encoded domain. *J Biol Chem*, 273, 20556-67 (1998)
51. Huang, J., J. Moore, S. Soffer, E. Kim, D. Rowe, C. A. Manley, K. O'Toole, W. Middlesworth, C. Stolar, D. Yamashiro & J. Kandel: Highly specific antiangiogenic therapy is effective in suppressing growth of experimental Wilms tumors. *J Pediatr Surg*, 36, 357-61 (2001)
52. Holash, J., S. Davis, N. Papadopoulos, S. D. Croll, L. Ho, M. Russell, P. Boland, R. Leidich, D. Hylton, E. Burova, E. Ioffe, T. Huang, C. Radziejewski, K. Bailey, J. P. Fandl, T. Daly, S. J. Wiegand, G. D. Yancopoulos & J. S. Rudge: VEGF-Trap: a VEGF blocker with potent antitumor effects. *Proc Natl Acad Sci U S A*, 99, 11393-8 (2002)
53. Kim, E. S., A. Serur, J. Huang, C. A. Manley, K. W. McCrudden, J. S. Frischer, S. Z. Soffer, L. Ring, T. New, S. Zabski, J. S. Rudge, J. Holash, G. D. Yancopoulos, J. J. Kandel & D. J. Yamashiro: Potent VEGF blockade causes regression of coopted vessels in a model of neuroblastoma. *Proc Natl Acad Sci U S A*, 99, 11399-404 (2002)
54. Granato, A. M., O. Nanni, F. Falcini, S. Folli, G. Mosconi, F. De Paola, L. Medri, D. Amadori & A. Volpi: Basic fibroblast growth factor and vascular endothelial growth factor serum levels in breast cancer patients and healthy women: useful as diagnostic tools? *Breast Cancer Res*, 6, R38-45 (2004)
55. Jellinek, D., C. K. Lynott, D. B. Rifkin & N. Janjic: High-affinity RNA ligands to basic fibroblast growth factor inhibit receptor binding. *Proc Natl Acad Sci U S A*, 90, 11227-31 (1993)
56. Golden, M. C., B. D. Collins, M. C. Willis & T. H. Koch: Diagnostic potential of PhotoSELEX-evolved ssDNA aptamers. *J Biotechnol*, 81, 167-78 (2000)
57. Golden, M. C., K. A. Resing, B. D. Collins, M. C. Willis & T. H. Koch: Mass spectral characterization of a protein-nucleic acid photocrosslink. *Protein Sci*, 8, 2806-12 (1999)
58. Jensen, K. B., B. L. Atkinson, M. C. Willis, T. H. Koch & L. Gold: Using *in vitro* selection to direct the covalent attachment of human immunodeficiency virus type 1 Rev protein to high-affinity RNA ligands. *Proc Natl Acad Sci U S A*, 92, 12220-4 (1995)
59. Lupold, S. E., B. J. Hicke, Y. Lin & D. S. Coffey: Identification and characterization of nuclease-stabilized RNA molecules that bind human prostate cancer cells via the prostate-specific membrane antigen. *Cancer Res*, 62, 4029-33 (2002)
60. Farokhzad, O. C., S. Jon, A. Khademhosseini, T. N. Tran, D. A. Lavan & R. Langer: Nanoparticle-aptamer bioconjugates: a new approach for targeting prostate cancer cells. *Cancer Res*, 64, 7668-72 (2004)
61. Smith, D., B. D. Collins, J. Heil & T. H. Koch: Sensitivity and specificity of photoaptamer probes. *Mol Cell Proteomics*, 2, 11-8 (2003)
62. Bock, C., M. Coleman, B. Collins, J. Davis, G. Foulds, L. Gold, C. Greef, J. Heil, J. S. Heilig, B. Hicke, M. N. Hurst, G. M. Husar, D. Miller, R. Ostroff, H. Petach, D. Schneider, B. Vant-Hull, S. Waugh, A. Weiss, S. K. Wilcox & D. Zichi: Photoaptamer arrays applied to multiplexed proteomic analysis. *Proteomics*, 4, 609-18 (2004)

63. Mocellin, S., C. R. Rossi & D. Nitti: Cancer vaccine development: on the way to break immune tolerance to malignant cells. *Exp Cell Res*, 299, 267-78 (2004)
64. Bradbury, A. R. & J. D. Marks: Antibodies from phage antibody libraries. *J Immunol Methods*, 290, 29-49 (2004)
65. Rader, C. & C. F. Barbas, 3rd: Phage display of combinatorial antibody libraries. *Curr Opin Biotechnol*, 8, 503-8 (1997)
66. Morris, K. N., K. B. Jensen, C. M. Julin, M. Weil & L. Gold: High affinity ligands from *in vitro* selection: complex targets. *Proc Natl Acad Sci U S A*, 95, 2902-7 (1998)
67. Blank, M., T. Weinschenk, M. Priemer & H. Schluesener: Systematic evolution of a DNA aptamer binding to rat brain tumor microvessels. selective targeting of endothelial regulatory protein pigpen. *J Biol Chem*, 276, 16464-8 (2001)
68. Alliegro, M. C. & M. A. Alliegro: Nuclear injection of anti-pigpen antibodies inhibits endothelial cell division. *J Biol Chem*, 277, 19037-41 (2002)
69. Wang, C., M. Zhang, G. Yang, D. Zhang, H. Ding, H. Wang, M. Fan, B. Shen & N. Shao: Single-stranded DNA aptamers that bind differentiated but not parental cells: subtractive systematic evolution of ligands by exponential enrichment. *J Biotechnol*, 102, 15-22 (2003)
70. Hicke, B. J., C. Marion, Y. F. Chang, T. Gould, C. K. Lynott, D. Parma, P. G. Schmidt & S. Warren: Tenascin-C aptamers are generated using tumor cells and purified protein. *J Biol Chem*, 276, 48644-54 (2001)
71. Daniels, D. A., H. Chen, B. J. Hicke, K. M. Swiderek & L. Gold: A tenascin-C aptamer identified by tumor cell SELEX: systematic evolution of ligands by exponential enrichment. *Proc Natl Acad Sci U S A*, 100, 15416-21 (2003)
72. Tsai, D. E. & J. D. Keene: *In vitro* selection of RNA epitopes using autoimmune patient serum. *J Immunol*, 150, 1137-45 (1993)
73. Lee, S. W. & B. A. Sullenger: Isolation of a nuclease-resistant decoy RNA that can protect human acetylcholine receptors from myasthenic antibodies. *Nat Biotechnol*, 15, 41-5 (1997)
74. Hwang, B. & S. W. Lee: Improvement of RNA aptamer activity against myasthenic autoantibodies by extended sequence selection. *Biochem Biophys Res Commun*, 290, 656-62 (2002)
75. Hwang, B., K. Han & S. W. Lee: Prevention of passively transferred experimental autoimmune myasthenia gravis by an *in vitro* selected RNA aptamer. *FEBS Lett*, 548, 85-9 (2003)
76. Rhodes, A., A. Deakin, J. Spaull, B. Coomber, A. Aitken, P. Life & S. Rees: The generation and characterization of antagonist RNA aptamers to human oncostatin M. *J Biol Chem*, 275, 28555-61 (2000)
77. Hicke, B. J., S. R. Watson, A. Koenig, C. K. Lynott, R. F. Bargatze, Y. F. Chang, S. Ringquist, L. Moon-McDermott, S. Jennings, T. Fitzwater, H. L. Han, N. Varki, I. Albinana, M. C. Willis, A. Varki & D. Parma: DNA aptamers block L-selectin function *in vivo*. Inhibition of human lymphocyte trafficking in SCID mice. *J Clin Invest*, 98, 2688-92 (1996)
78. Jeong, S., T. Eom, S. Kim, S. Lee & J. Yu: *In vitro* selection of the RNA aptamer against the Sialyl Lewis X and its inhibition of the cell adhesion. *Biochem Biophys Res Commun*, 281, 237-43 (2001)
79. Lewis, T. S., P. S. Shapiro & N. G. Ahn: Signal transduction through MAP kinase cascades. *Adv Cancer Res*, 74, 49-139 (1998)
80. Kimoto, M., M. Shirouzu, S. Mizutani, H. Koide, Y. Kaziro, I. Hirao & S. Yokoyama: Anti- (Raf-1) RNA aptamers that inhibit Ras-induced Raf-1 activation. *Eur J Biochem*, 269, 697-704 (2002)
81. Blind, M., W. Kolanus & M. Famulok: Cytoplasmic RNA modulators of an inside-out signal-transduction cascade. *Proc Natl Acad Sci U S A*, 96, 3606-10 (1999)
82. Mayer, G., M. Blind, W. Nagel, T. Bohm, T. Knorr, C. L. Jackson, W. Kolanus & M. Famulok: Controlling small guanine-nucleotide-exchange factor function through cytoplasmic RNA intramers. *Proc Natl Acad Sci U S A*, 98, 4961-5 (2001)
83. Tuerk, C. & L. Gold: Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science*, 249, 505-10 (1990)
84. Thomas, M., S. Chedin, C. Carles, M. Riva, M. Famulok & A. Sentenac: Selective targeting and inhibition of yeast RNA polymerase II by RNA aptamers. *J Biol Chem*, 272, 27980-6 (1997)
85. Baeuerle, P. A. & D. Baltimore: NF-kappa B: ten years after. *Cell*, 87, 13-20 (1996)
86. Morishita, R., T. Sugimoto, M. Aoki, I. Kida, N. Tomita, A. Moriguchi, K. Maeda, Y. Sawa, Y. Kaneda, J. Higaki & T. Ogihara: *In vivo* transfection of cis element "decoy" against nuclear factor-kappaB binding site prevents myocardial infarction. *Nat Med*, 3, 894-9 (1997)
87. Beg, A. A. & D. Baltimore: An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. *Science*, 274, 782-4 (1996)
88. Lebruska, L. L. & L. J. Maher, 3rd: Selection and characterization of an RNA decoy for transcription factor NF-kappa B. *Biochemistry*, 38, 3168-74 (1999)
89. Cassiday, L. A. & L. J. Maher, 3rd: *In vivo* recognition of an RNA aptamer by its transcription factor target. *Biochemistry*, 40, 2433-8 (2001)
90. Cassiday, L. A. & L. J. Maher, 3rd: Yeast genetic selections to optimize RNA decoys for transcription factor NF-kappa B. *Proc Natl Acad Sci U S A*, 100, 3930-5 (2003)
91. Shi, H., B. E. Hoffman & J. T. Lis: A specific RNA hairpin loop structure binds the RNA recognition motifs of the Drosophila SR protein B52. *Mol Cell Biol*, 17, 2649-57 (1997)
92. Shi, H., B. E. Hoffman & J. T. Lis: RNA aptamers as effective protein antagonists in a multicellular organism. *Proc Natl Acad Sci U S A*, 96, 10033-8 (1999)
93. Eckner, R., W. Ellmeier & M. L. Birnstiel: Mature mRNA 3' end formation stimulates RNA export from the nucleus. *Embo J*, 10, 3513-22 (1991)
94. Liu, Z., D. B. Batt & G. G. Carmichael: Targeted nuclear antisense RNA mimics natural antisense-induced degradation of polyoma virus early RNA. *Proc Natl Acad Sci U S A*, 91, 4258-62 (1994)
95. Furth, P. A., A. Shamay & L. Hennighausen: Gene transfer into mammalian cells by jet injection. *Hybridoma*, 14, 149-52 (1995)
96. Liu, F., Y. Song & D. Liu: Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther*, 6, 1258-66 (1999)
97. Leppanen, O., N. Janjic, M. A. Carlsson, K. Pietras, M. Levin, C. Vargeese, L. S. Green, D. Bergqvist, A. Ostman

& C. H. Heldin: Intimal hyperplasia recurs after removal of PDGF-AB and -BB inhibition in the rat carotid artery injury model. *Arterioscler Thromb Vasc Biol*, 20, E89-95 (2000)
98. Bock, L. C., L. C. Griffin, J. A. Latham, E. H. Vermaas & J. J. Toole: Selection of single-stranded DNA molecules that bind and inhibit human thrombin. *Nature*, 355, 564-6 (1992)
99. DeAnda, A., Jr., S. E. Coutre, M. R. Moon, C. M. Vial, L. C. Griffin, V. S. Law, M. Komeda, L. L. Leung & D. C. Miller: Pilot study of the efficacy of a thrombin inhibitor for use during cardiopulmonary bypass. *Ann Thorac Surg*, 58, 344-50 (1994)
100. Griffin, L. C., G. F. Tidmarsh, L. C. Bock, J. J. Toole & L. L. Leung: *In vivo* anticoagulant properties of a novel nucleotide-based thrombin inhibitor and demonstration of regional anticoagulation in extracorporeal circuits. *Blood*, 81, 3271-6 (1993)
101. Bless, N. M., D. Smith, J. Charlton, B. J. Czermak, H. Schmal, H. P. Friedl & P. A. Ward: Protective effects of an aptamer inhibitor of neutrophil elastase in lung inflammatory injury. *Curr Biol*, 7, 877-80 (1997)
102. Drolet, D. W., J. Nelson, C. E. Tucker, P. M. Zack, K. Nixon, R. Bolin, M. B. Judkins, J. A. Farmer, J. L. Wolf, S. C. Gill & R. A. Bendele: Pharmacokinetics and safety of an anti-vascular endothelial growth factor aptamer (NX1838) following injection into the vitreous humor of rhesus monkeys. *Pharm Res*, 17, 1503-10 (2000)
103. Anti-vascular endothelial growth factor therapy for subfoveal choroidal neovascularization secondary to age-related macular degeneration: phase II study results. *Ophthalmology*, 110, 979-86 (2003)
104. Lassus, P., M. Turanlahti, P. Heikkila, L. C. Andersson, I. Nupponen, A. Sarnesto & S. Andersson: Pulmonary vascular endothelial growth factor and Flt-1 in fetuses, in acute and chronic lung disease, and in persistent pulmonary hypertension of the newborn. *Am J Respir Crit Care Med*, 164, 1981-7 (2001)
105. Partovian, C., S. Adnot, S. Eddahibi, E. Teiger, M. Levame, P. Dreyfus, B. Raffestin & C. Frelin: Heart and lung VEGF mRNA expression in rats with monocrotaline- or hypoxia-induced pulmonary hypertension. *Am J Physiol*, 275, H1948-56 (1998)
106. Grover, T. R., T. A. Parker, J. P. Zenge, N. E. Markham, J. P. Kinsella & S. H. Abman: Intrauterine hypertension decreases lung VEGF expression and VEGF inhibition causes pulmonary hypertension in the ovine fetus. *Am J Physiol Lung Cell Mol Physiol*, 284, L508-17 (2003)
107. Ishizaki, J., J. R. Nevins & B. A. Sullenger: Inhibition of cell proliferation by an RNA ligand that selectively blocks E2F function. *Nat Med*, 2, 1386-9 (1996)
108. Carrasquillo, K. G., J. A. Ricker, I. K. Rigas, J. W. Miller, E. S. Gragoudas & A. P. Adamis: Controlled delivery of the anti-VEGF aptamer EYE001 with poly (lactic-co-glycolic)acid microspheres. *Invest Ophthalmol Vis Sci*, 44, 290-9 (2003)
109. White, R. R., S. Shan, C. P. Rusconi, G. Shetty, M. W. Dewhirst, C. D. Kontos & B. A. Sullenger: Inhibition of rat corneal angiogenesis by a nuclease-resistant RNA aptamer specific for angiopoietin-2. *Proc Natl Acad Sci U S A*, 100, 5028-33 (2003)
110. Agrawal, S., X. Zhang, Z. Lu, H. Zhao, J. M. Tamburin, J. Yan, H. Cai, R. B. Diasio, I. Habus, Z. Jiang & *et al.*: Absorption, tissue distribution and *in vivo* stability in rats of a hybrid antisense oligonucleotide following oral administration. *Biochem Pharmacol*, 50, 571-6 (1995)
111. Dougan, H., D. M. Lyster, C. V. Vo, A. Stafford, J. I. Weitz & J. B. Hobbs: Extending the lifetime of anticoagulant oligodeoxynucleotide aptamers in blood. *Nucl Med Biol*, 27, 289-97 (2000)
112. Dachs, G. U., G. J. Dougherty, I. J. Stratford & D. J. Chaplin: Targeting gene therapy to cancer: a review. *Oncol Res*, 9, 313-25 (1997)
113. Cristiano, R. J. & J. A. Roth: Epidermal growth factor mediated DNA delivery into lung cancer cells via the epidermal growth factor receptor. *Cancer Gene Ther*, 3, 4-10 (1996)
114. Gottschalk, S., R. J. Cristiano, L. C. Smith & S. L. Woo: Folate receptor mediated DNA delivery into tumor cells: potosomal disruption results in enhanced gene expression. *Gene Ther*, 1, 185-91 (1994)
115. Lee, R. J. & L. Huang: Folate-targeted, anionic liposome-entrapped polylysine-condensed DNA for tumor cell-specific gene transfer. *J Biol Chem*, 271, 8481-7 (1996)
116. Li, S., H. M. Deshmukh & L. Huang: Folate-mediated targeting of antisense oligodeoxynucleotides to ovarian cancer cells. *Pharm Res*, 15, 1540-5 (1998)
117. Israeli, R. S., C. T. Powell, J. G. Corr, W. R. Fair & W. D. Heston: Expression of the prostate-specific membrane antigen. *Cancer Res*, 54, 1807-11 (1994)
118. Charlton, J., G. P. Kirschenheuter & D. Smith: Highly potent irreversible inhibitors of neutrophil elastase generated by selection from a randomized DNA-valine phosphonate library. *Biochemistry*, 36, 3018-26 (1997)
119. Smith, D., G. P. Kirschenheuter, J. Charlton, D. M. Guidot & J. E. Repine: *In vitro* selection of RNA-based irreversible inhibitors of human neutrophil elastase. *Chem Biol*, 2, 741-50 (1995)
120. Hicke, B. J. & A. W. Stephens: Escort aptamers: a delivery service for diagnosis and therapy. *J Clin Invest*, 106, 923-8 (2000)
121. Hilger, C. S., M. C. Willis, M. Wolters & W. A. Pieken: Tc-99m-labeling of modified RNA. *Nucleosides Nucleotides*, 18, 1479-81 (1999)
122. Brody, E. N. & L. Gold: Aptamers as therapeutic and diagnostic agents. *J Biotechnol*, 74, 5-13 (2000)
123. Cerchia, L., J. Hamm, D. Libri, B. Tavitian & V. de Franciscis: Nucleic acid aptamers in cancer medicine. *FEBS Lett*, 528, 12-6 (2002)
124. Jellinek, D., L. S. Green, C. Bell, C. K. Lynott, N. Gill, C. Vargeese, G. Kirschenheuter, D. P. McGee, P. Abesinghe, W. A. Pieken & *et al.*: Potent 2'-amino-2'-deoxypyrimidine RNA inhibitors of basic fibroblast growth factor. *Biochemistry*, 34, 11363-72 (1995)
125. Pieken, W. A., D. B. Olsen, F. Benseler, H. Aurup & F. Eckstein: Kinetic characterization of ribonuclease-resistant 2'-modified hammerhead ribozymes. *Science*, 253, 314-7 (1991)
126. Chelliserrykattil, J. & A. D. Ellington: Evolution of a T7 RNA polymerase variant that transcribes 2'-O-methyl RNA. *Nat Biotechnol*, 22, 1155-60 (2004)
127. Lee, W. A., J. A. Fishback, J. P. Shaw, L. C. Bock, L. C. Griffin & K. C. Cundy: A novel oligodeoxynucleotide

inhibitor of thrombin. II. Pharmacokinetics in the cynomolgus monkey. *Pharm Res*, 12, 1943-7 (1995)
128. Tucker, C. E., L. S. Chen, M. B. Judkins, J. A. Farmer, S. C. Gill & D. W. Drolet: Detection and plasma pharmacokinetics of an anti-vascular endothelial growth factor oligonucleotide-aptamer (NX1838) in rhesus monkeys. *J Chromatogr B Biomed Sci Appl*, 732, 203-12 (1999)
129. Watson, S. R., Y. F. Chang, D. O'Connell, L. Weigand, S. Ringquist & D. H. Parma: Anti-L-selectin aptamers: binding characteristics, pharmacokinetic parameters, and activity against an intravascular target *in vivo*. *Antisense Nucleic Acid Drug Dev*, 10, 63-75 (2000)
130. de Smidt, P. C., T. Le Doan, S. de Falco & T. J. van Berkel: Association of antisense oligonucleotides with lipoproteins prolongs the plasma half-life and modifies the tissue distribution. *Nucleic Acids Res*, 19, 4695-700 (1991)
131. Willis, M. C., B. D. Collins, T. Zhang, L. S. Green, D. P. Sebesta, C. Bell, E. Kellogg, S. C. Gill, A. Magallanez, S. Knauer, R. A. Bendele, P. S. Gill, N. Janjic & B. Collins: Liposome-anchored vascular endothelial growth factor aptamers. *Bioconjug Chem*, 9, 573-82 (1998)
132. Rusconi, C. P., E. Scardino, J. Layzer, G. A. Pitoc, T. L. Ortel, D. Monroe & B. A. Sullenger: RNA aptamers as reversible antagonists of coagulation factor IXa. *Nature*, 419, 90-4 (2002)
133. Porsche, R. & Z. R. Brenner: Allergy to protamine sulfate. *Heart Lung*, 28, 418-28 (1999)
134. Rusconi, C. P., J. D. Roberts, G. A. Pitoc, S. M. Nimjee, R. R. White, G. Quick, Jr., E. Scardino, W. P. Fay & B. A. Sullenger: Antidote-mediated control of an anticoagulant aptamer *in vivo*. *Nat Biotechnol*, 22, 1423-8 (2004)
135. Robbins, P. D., H. Tahara & S. C. Ghivizzani: Viral vectors for gene therapy. *Trends Biotechnol*, 16, 35-40 (1998)
136. Walther, W. & U. Stein: Viral vectors for gene transfer: a review of their use in the treatment of human diseases. *Drugs*, 60, 249-71 (2000)
137. Li, S. & L. Huang: Nonviral gene therapy: promises and challenges. *Gene Ther*, 7, 31-4 (2000)
138. Torchilin, V. P., B. A. Khaw & V. Weissig: Intracellular targets for DNA delivery: nuclei and mitochondria. *Somat Cell Mol Genet*, 27, 49-64 (2002)
139. Tranchant, I., B. Thompson, C. Nicolazzi, N. Mignet & D. Scherman: Physicochemical optimisation of plasmid delivery by cationic lipids. *J Gene Med*, 6 Suppl 1, S24-35 (2004)
140. Kumar, V. V., R. S. Singh & A. Chaudhuri: Cationic transfection lipids in gene therapy: successes, set-backs, challenges and promises. *Curr Med Chem*, 10, 1297-306 (2003)
141. Miller, A. D.: The problem with cationic liposome/micelle-based non-viral vector systems for gene therapy. *Curr Med Chem*, 10, 1195-211 (2003)
142. Nabel, G. J., E. G. Nabel, Z. Y. Yang, B. A. Fox, G. E. Plautz, X. Gao, L. Huang, S. Shu, D. Gordon & A. E. Chang: Direct gene transfer with DNA-liposome complexes in melanoma: expression, biologic activity, and lack of toxicity in humans. *Proc Natl Acad Sci U S A*, 90, 11307-11 (1993)
143. Caplen, N. J., E. W. Alton, P. G. Middleton, J. R. Dorin, B. J. Stevenson, X. Gao, S. R. Durham, P. K. Jeffery, M. E. Hodson, C. Coutelle & *et al.*: Liposome-mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis. *Nat Med*, 1, 39-46 (1995)
144. Stopeck, A. T., E. M. Hersh, E. T. Akporiaye, D. T. Harris, T. Grogan, E. Unger, J. Warneke, S. F. Schluter & S. Stahl: Phase I study of direct gene transfer of an allogeneic histocompatibility antigen, HLA-B7, in patients with metastatic melanoma. *J Clin Oncol*, 15, 341-9 (1997)
145. Moghimi, S. M. & J. Szebeni: Stealth liposomes and long circulating nanoparticles: critical issues in pharmacokinetics, opsonization and protein-binding properties. *Prog Lipid Res*, 42, 463-78 (2003)
146. Nicolazzi, C., N. Mignet, N. de la Figuera, M. Cadet, R. T. Ibad, J. Seguin, D. Scherman & M. Bessodes: Anionic polyethyleneglycol lipids added to cationic lipoplexes increase their plasmatic circulation time. *J Control Release*, 88, 429-43 (2003)
147. Li, S. & Z. Ma: Nonviral gene therapy. *Curr Gene Ther*, 1, 201-26 (2001)
148. Pedroso de Lima, M. C., S. Neves, A. Filipe, N. Duzgunes & S. Simoes: Cationic liposomes for gene delivery: from biophysics to biological applications. *Curr Med Chem*, 10, 1221-31 (2003)
149. Zuhorn, I. S. & D. Hoekstra: On the mechanism of cationic amphiphile-mediated transfection. To fuse or not to fuse: is that the question? *J Membr Biol*, 189, 167-79 (2002)
150. Audouy, S. & D. Hoekstra: Cationic lipid-mediated transfection *in vitro* and *in vivo* (review). *Mol Membr Biol*, 18, 129-43 (2001)
151. Duzgunes, N., C. T. De Ilarduya, S. Simoes, R. I. Zhdanov, K. Konopka & M. C. Pedroso de Lima: Cationic liposomes for gene delivery: novel cationic lipids and enhancement by proteins and peptides. *Curr Med Chem*, 10, 1213-20 (2003)
152. Tros de Ilarduya, C., M. A. Arangoa & N. Duzgunes: Transferrin-lipoplexes with protamine-condensed DNA for serum-resistant gene delivery. *Methods Enzymol*, 373, 342-56 (2003)
153. Tros de Ilarduya, C., M. A. Arangoa, M. J. Moreno-Aliaga & N. Duzgunes: Enhanced gene delivery *in vitro* and *in vivo* by improved transferrin-lipoplexes. *Biochim Biophys Acta*, 1561, 209-21 (2002)
154. Erbacher, P., T. Bettinger, P. Belguise-Valladier, S. Zou, J. L. Coll, J. P. Behr & J. S. Remy: Transfection and physical properties of various saccharide, poly (ethylene glycol), and antibody-derivatized polyethylenimines (PEI). *J Gene Med*, 1, 210-22 (1999)
155. Godbey, W. T., K. K. Wu & A. G. Mikos: Poly (ethylenimine) and its role in gene delivery. *J Control Release*, 60, 149-60 (1999)
156. Lemkine, G. F. & B. A. Demeneix: Polyethylenimines for *in vivo* gene delivery. *Curr Opin Mol Ther*, 3, 178-82 (2001)
157. Fini, A. & I. Orienti: The role of chitosan in drug delivery: current and potential applications. *Am J Drug Delivery*, 1, 43-59 (2003)
158. Vijayanathan, V., T. Thomas & T. J. Thomas: DNA nanoparticles and development of DNA delivery vehicles for gene therapy. *Biochemistry*, 41, 14085-94 (2002)
159. Panyam, J. & V. Labhasetwar: Biodegradable nanoparticles for drug and gene delivery to cells and tissue. *Adv Drug Deliv Rev*, 55, 329-47 (2003)

160. Muller, R. H., K. Mader & S. Gohla: Solid lipid nanoparticles (SLN) for controlled drug delivery - a review of the state of the art. *Eur J Pharm Biopharm*, 50, 161-77 (2000)
161. Ballou, B., B. C. Lagerholm, L. A. Ernst, M. P. Bruchez & A. S. Waggoner: Noninvasive imaging of quantum dots in mice. *Bioconjug Chem*, 15, 79-86 (2004)
162. Gao, X., Y. Cui, R. M. Levenson, L. W. Chung & S. Nie: *In vivo* cancer targeting and imaging with semiconductor quantum dots. *Nat Biotechnol*, 22, 969-76 (2004)
163. Mir, L. M., M. F. Bureau, J. Gehl, R. Rangara, D. Rouy, J. M. Caillaud, P. Delaere, D. Branellec, B. Schwartz & D. Scherman: High-efficiency gene transfer into skeletal muscle mediated by electric pulses. *Proc Natl Acad Sci U S A*, 96, 4262-7 (1999)
164. Gehl, J.: Electroporation: theory and methods, perspectives for drug delivery, gene therapy and research. *Acta Physiol Scand*, 177, 437-47 (2003)
165. Gresch, O., F. B. Engel, D. Nesic, T. T. Tran, H. M. England, E. S. Hickman, I. Korner, L. Gan, S. Chen, S. Castro-Obregon, R. Hammermann, J. Wolf, H. Muller-Hartmann, M. Nix, G. Siebenkotten, G. Kraus & K. Lun: New non-viral method for gene transfer into primary cells. *Methods*, 33, 151-63 (2004)
166. Kuriyama, S., A. Mitoro, H. Tsujinoue, T. Nakatani, H. Yoshiji, T. Tsujimoto, M. Yamazaki & H. Fukui: Particle-mediated gene transfer into murine livers using a newly developed gene gun. *Gene Ther*, 7, 1132-6 (2000)
167. Nishizaki, K., O. Mazda, Y. Dohi, T. Kawata, K. Mizuguchi, S. Kitamura & S. Taniguchi: *In vivo* gene gun-mediated transduction into rat heart with Epstein-Barr virus-based episomal vectors. *Ann Thorac Surg*, 70, 1332-7 (2000)
168. Jackson, D. A., A. Pombo & F. Iborra: The balance sheet for transcription: an analysis of nuclear RNA metabolism in mammalian cells. *Faseb J*, 14, 242-54 (2000)
169. Paule, M. R. & R. J. White: Survey and summary: transcription by RNA polymerases I and III. *Nucleic Acids Res*, 28, 1283-98 (2000)
170. Pang, S., S. Taneja, K. Dardashti, P. Cohan, R. Kaboo, M. Sokoloff, C. L. Tso, J. B. Dekernion & A. S. Belldegrun: Prostate tissue specificity of the prostate-specific antigen promoter isolated from a patient with prostate cancer. *Hum Gene Ther*, 6, 1417-26 (1995)
171. Ko, S. C., J. Cheon, C. Kao, A. Gotoh, T. Shirakawa, R. A. Sikes, G. Karsenty & L. W. Chung: Osteocalcin promoter-based toxic gene therapy for the treatment of osteosarcoma in experimental models. *Cancer Res*, 56, 4614-9 (1996)
172. Stein, G. S., J. B. Lian, J. L. Stein & A. J. van Wijnen: Bone tissue specific transcriptional control: options for targeting gene therapy to the skeleton. *Cancer*, 88, 2899-902 (2000)
173. Kuriyama, S., M. Yoshikawa, S. Ishizaka, T. Tsujii, K. Ikenaka, T. Kagawa, N. Morita & K. Mikoshiba: A potential approach for gene therapy targeting hepatoma using a liver-specific promoter on a retroviral vector. *Cell Struct Funct*, 16, 503-10 (1991)
174. Macri, P. & J. W. Gordon: Delayed morbidity and mortality of albumin/SV40 T-antigen transgenic mice after insertion of an alpha-fetoprotein/herpes virus thymidine kinase transgene and treatment with ganciclovir. *Hum Gene Ther*, 5, 175-82 (1994)
175. Hewitt, S. M., G. C. Fraizer, Y. J. Wu, F. J. Rauscher, 3rd & G. F. Saunders: Differential function of Wilms' tumor gene WT1 splice isoforms in transcriptional regulation. *J Biol Chem*, 271, 8588-92 (1996)
176. Ozaki, K., T. Yoshida, H. Ide, I. Saito, Y. Ikeda, T. Sugimura & M. Terada: Use of von Willebrand factor promoter to transduce suicidal gene to human endothelial cells, HUVEC. *Hum Gene Ther*, 7, 1483-90 (1996)
177. Vile, R. G. & I. R. Hart: Use of tissue-specific expression of the herpes simplex virus thymidine kinase gene to inhibit growth of established murine melanomas following direct intratumoral injection of DNA. *Cancer Res*, 53, 3860-4 (1993)
178. Miyao, Y., K. Shimizu, S. Moriuchi, M. Yamada, K. Nakahira, K. Nakajima, J. Nakao, S. Kuriyama, T. Tsujii, K. Mikoshiba & *et al.*: Selective expression of foreign genes in glioma cells: use of the mouse myelin basic protein gene promoter to direct toxic gene expression. *J Neurosci Res*, 36, 472-9 (1993)
179. O'Keefe, D. S., A. Uchida, D. J. Bacich, F. B. Watt, A. Martorana, P. L. Molloy & W. D. Heston: Prostate-specific suicide gene therapy using the prostate-specific membrane antigen promoter and enhancer. *Prostate*, 45, 149-57 (2000)
180. Sullenger, B. A., T. C. Lee, C. A. Smith, G. E. Ungers & E. Gilboa: Expression of chimeric tRNA-driven antisense transcripts renders NIH 3T3 cells highly resistant to Moloney murine leukemia virus replication. *Mol Cell Biol*, 10, 6512-23 (1990)
181. Lee, S. W., H. F. Gallardo, O. Gaspar, C. Smith & E. Gilboa: Inhibition of HIV-1 in CEM cells by a potent TAR decoy. *Gene Ther*, 2, 377-84 (1995)
182. Hantzopoulos, P. A., B. A. Sullenger, G. Ungers & E. Gilboa: Improved gene expression upon transfer of the adenosine deaminase minigene outside the transcriptional unit of a retroviral vector. *Proc Natl Acad Sci U S A*, 86, 3519-23 (1989)
183. Bertrand, E., D. Castanotto, C. Zhou, C. Carbonnelle, N. S. Lee, P. Good, S. Chatterjee, T. Grange, R. Pictet, D. Kohn, D. Engelke & J. J. Rossi: The expression cassette determines the functional activity of ribozymes in mammalian cells by controlling their intracellular localization. *Rna*, 3, 75-88 (1997)
184. Chaloin, L., M. J. Lehmann, G. Sczakiel & T. Restle: Endogenous expression of a high-affinity pseudoknot RNA aptamer suppresses replication of HIV-1. *Nucleic Acids Res*, 30, 4001-8 (2002)
185. Kechli, A. M., P. J. Freiden, J. J. Rossi, M. K. Brenner, M. A. Choueiry, J. V. Garcia & K. S. Slobod: Expression of the human immunodeficiency virus type 1 primer binding sequence inhibits HIV-1 replication. *Hum Gene Ther*, 9, 587-90 (1998)
186. Martell, R. E., J. R. Nevins & B. A. Sullenger: Optimizing aptamer activity for gene therapy applications using expression cassette SELEX. *Mol Ther*, 6, 30-4 (2002)
187. Werstuck, G. & M. R. Green: Controlling gene expression in living cells through small molecule-RNA interactions. *Science*, 282, 296-8 (1998)
188. Suess, B., S. Hanson, C. Berens, B. Fink, R. Schroeder & W. Hillen: Conditional gene expression by controlling

translation with tetracycline-binding aptamers. *Nucleic Acids Res*, 31, 1853-8 (2003)
189. Grate, D. & C. Wilson: Inducible regulation of the S. cerevisiae cell cycle mediated by an RNA aptamer-ligand complex. *Bioorg Med Chem*, 9, 2565-70 (2001)
190. Winkler, W., A. Nahvi & R. R. Breaker: Thiamine derivatives bind messenger RNAs directly to regulate bacterial gene expression. *Nature*, 419, 952-6 (2002)
191. Brantl, S.: Bacterial gene regulation: from transcription attenuation to riboswitches and ribozymes. *Trends Microbiol*, 12, 473-5 (2004)
192. Nudler, E. & A. S. Mironov: The riboswitch control of bacterial metabolism. *Trends Biochem Sci*, 29, 11-7 (2004)
193. Vitreschak, A. G., D. A. Rodionov, A. A. Mironov & M. S. Gelfand: Riboswitches: the oldest mechanism for the regulation of gene expression? *Trends Genet*, 20, 44-50 (2004)
194. Winkler, W. C. & R. R. Breaker: Genetic control by metabolite-binding riboswitches. *Chembiochem*, 4, 1024-32 (2003)
195. Buskirk, A. R., P. D. Kehayova, A. Landrigan & D. R. Liu: *In vivo* evolution of an RNA-based transcriptional activator. *Chem Biol*, 10, 533-40 (2003)
196. Soukup, G. A. & R. R. Breaker: Allosteric nucleic acid catalysts. *Curr Opin Struct Biol*, 10, 318-25 (2000)
197. Suess, B., B. Fink, C. Berens, R. Stentz & W. Hillen: A theophylline responsive riboswitch based on helix slipping controls gene expression *in vivo*. *Nucleic Acids Res*, 32, 1610-4 (2004)
198. Vuyisich, M. & P. A. Beal: Controlling protein activity with ligand-regulated RNA aptamers. *Chem Biol*, 9, 907-13 (2002)
199. Allen, P., S. Worland & L. Gold: Isolation of high-affinity RNA ligands to HIV-1 integrase from a random pool. *Virology*, 209, 327-36 (1995)
200. Giver, L., D. P. Bartel, M. L. Zapp, M. R. Green & A. D. Ellington: Selection and design of high-affinity RNA ligands for HIV-1 Rev. *Gene*, 137, 19-24 (1993)
201. Berglund, J. A., B. Charpentier & M. Rosbash: A high affinity binding site for the HIV-1 nucleocapsid protein. *Nucleic Acids Res*, 25, 1042-9 (1997)
202. Tuerk, C. & S. MacDougal-Waugh: *In vitro* evolution of functional nucleic acids: high-affinity RNA ligands of HIV-1 proteins. *Gene*, 137, 33-9 (1993)
203. Yamamoto, R., M. Katahira, S. Nishikawa, T. Baba, K. Taira & P. K. Kumar: A novel RNA motif that binds efficiently and specifically to the Ttat protein of HIV and inhibits the trans-activation by Tat of transcription *in vitro* and *in vivo*. *Genes Cells*, 5, 371-88 (2000)
204. Kubik, M. F., A. W. Stephens, D. Schneider, R. A. Marlar & D. Tasset: High-affinity RNA ligands to human alpha-thrombin. *Nucleic Acids Res*, 22, 2619-26 (1994)
205. Latham, J. A., R. Johnson & J. J. Toole: The application of a modified nucleotide in aptamer selection: novel thrombin aptamers containing 5- (1-pentynyl)-2'-deoxyuridine. *Nucleic Acids Res*, 22, 2817-22 (1994)
206. Gal, S. W., S. Amontov, P. T. Urvil, D. Vishnuvardhan, F. Nishikawa, P. K. Kumar & S. Nishikawa: Selection of a RNA aptamer that binds to human activated protein C and inhibits its protease function. *Eur J Biochem*, 252, 553-62 (1998)
207. Bell, S. D., J. M. Denu, J. E. Dixon & A. D. Ellington: RNA molecules that bind to and inhibit the active site of a tyrosine phosphatase. *J Biol Chem*, 273, 14309-14 (1998)
208. Xinrui Yan, X. G., and Zhiqing Zhang: Isolation and Characterization of 2'-amino-modified RNA Aptamers for Human TNF-alpha. *Genomics Proteomics Bioinformatics*, 2, 32-42 (2004)
209. Pagratis, N. C., C. Bell, Y. F. Chang, S. Jennings, T. Fitzwater, D. Jellinek & C. Dang: Potent 2'-amino-, and 2'-fluoro-2'-deoxyribonucleotide RNA inhibitors of keratinocyte growth factor. *Nat Biotechnol*, 15, 68-73 (1997)
210. Green, L. S., D. Jellinek, R. Jenison, A. Ostman, C. H. Heldin & N. Janjic: Inhibitory DNA ligands to platelet-derived growth factor B-chain. *Biochemistry*, 35, 14413-24 (1996)
211. Green, L. S., D. Jellinek, C. Bell, L. A. Beebe, B. D. Feistner, S. C. Gill, F. M. Jucker & N. Janjic: Nuclease-resistant nucleic acid ligands to vascular permeability factor/vascular endothelial growth factor. *Chem Biol*, 2, 683-95 (1995)
212. Cho, J. S., Y. J. Lee, K. S. Shin, S. Jeong, J. Park & S. W. Lee: *In vitro* selection of specific RNA aptamers for the NFAT DNA binding domain. *Mol Cells*, 18, 17-23 (2004)
213. Bardeesy, N. & J. Pelletier: Overlapping RNA and DNA binding domains of the wt1 tumor suppressor gene product. *Nucleic Acids Res*, 26, 1784-92 (1998)
214. Mori, T., A. Oguro, T. Ohtsu & Y. Nakamura: RNA aptamers selected against the receptor activator of NF-kappaB acquire general affinity to proteins of the tumor necrosis factor receptor family. *Nucleic Acids Res*, 32, 6120-8 (2004)
215. Chen, C. H., G. A. Chernis, V. Q. Hoang & R. Landgraf: Inhibition of heregulin signaling by an aptamer that preferentially binds to the oligomeric form of human epidermal growth factor receptor-3. *Proc Natl Acad Sci U S A*, 100, 9226-31 (2003)
216. O'Connell, D., A. Koenig, S. Jennings, B. Hicke, H. L. Han, T. Fitzwater, Y. F. Chang, N. Varki, D. Parma & A. Varki: Calcium-dependent oligonucleotide antagonists specific for L-selectin. *Proc Natl Acad Sci U S A*, 93, 5883-7 (1996)
217. Jenison, R. D., S. D. Jennings, D. W. Walker, R. F. Bargatze & D. Parma: Oligonucleotide inhibitors of P-selectin-dependent neutrophil-platelet adhesion. *Antisense Nucleic Acid Drug Dev*, 8, 265-79 (1998)
218. Bridonneau, P., Y. F. Chang, D. O'Connell, S. C. Gill, D. W. Snyder, L. Johnson, T. Goodson, Jr., D. K. Herron & D. H. Parma: High-affinity aptamers selectively inhibit human nonpancreatic secretory phospholipase A2 (hnps-PLA2). *J Med Chem*, 41, 778-86 (1998)
219. Wiegand, T. W., P. B. Williams, S. C. Dreskin, M. H. Jouvin, J. P. Kinet & D. Tasset: High-affinity oligonucleotide ligands to human IgE inhibit binding to Fc epsilon receptor I. *J Immunol*, 157, 221-30 (1996)
220. Kubik, M. F., C. Bell, T. Fitzwater, S. R. Watson & D. M. Tasset: Isolation and characterization of 2'-fluoro-, 2'-amino-, and 2'-fluoro-/amino-modified RNA ligands to human IFN-gamma that inhibit receptor binding. *J Immunol*, 159, 259-67 (1997)
221. Kraus, E., W. James & A. N. Barclay: Cutting edge: novel RNA ligands able to bind CD4 antigen and inhibit CD4+ T lymphocyte function. *J Immunol*, 160, 5209-12 (1998)
222. Biesecker, G., L. Dihel, K. Enney & R. A. Bendele: Derivation of RNA aptamer inhibitors of human complement C5. *Immunopharmacology*, 42, 219-30 (1999)

223Yoo, S. & W. S. Dynan: Characterization of the RNA binding properties of Ku protein. *Biochemistry*, 37, 1336-43 (1998)

Key words: Aptamers, Treatment, Therapeutics, Selex, Gene, Gene Therapy, Review

Send correspondence to: Dr. Andrew Ellington, University of Texas at Austin, 1 University Station A4800, Austin, TX, 78712-0159, Tel: 512-232-3424, Fax: 512-471-7014, E-mail: andy.ellington@mail.utexas.edu

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