Rapid selection of single-stranded DNA aptamers binding *Staphylococcus epidermidis* in platelet concentrates

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ABSTRACT

Staphylococcus epidermidis is the most common transfusion-associated pathogen contaminating platelet concentrates. Methods to reduce or eliminate contaminating bacteria from platelet units are critical for improving the safety of blood transfusions. We used rapid isolation of DNA aptamers (RIDA) to identify single-stranded (ss)DNA aptamers as ligands that specifically bind to *S. epidermidis*. Five target-specific ssDNA aptamers (76 mer) were obtained under stringent selection conditions. Aptamer SE43 demonstrated higher binding affinity compared with scrambled control. Furthermore, when binding assays were conducted in platelet concentrate, there was a twofold increase in binding affinity compared with the SE43 binding in buffer alone. Our data identified an aptamer that may be useful as a ligand to capture, detect or remove *S. epidermidis* contaminant from platelet concentrates.

KEYWORDS:

DNA aptamer • platelets • RIDA • SELEX

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METHOD SUMMARY

We used a one-step screening method to isolate single-stranded DNA aptamers that bound *Staphylococcus epidermidis*. One aptamer demonstrated higher binding affinity to *S. epidermidis* in platelet concentrates compared with scrambled control. These studies suggest that aptamers might be used to capture and remove bacteria contaminating platelet concentrates, thus improving the safety of platelets transfusion.

INTRODUCTION

Aptamers are a new class of affinity ligand that offer significant advantages compared with antibodies, including smaller size, greater stability, lack of immunogenicity, and ease of production by chemical synthesis [1,2]. Aptamers are single-stranded (ss)DNA or RNA molecules, typically 25–100 nucleotides, which fold into a well-defined secondary structure. They bind a variety of targets such as proteins, polypeptides, metal ions and whole cells with high affinity (nM to pM range), specificity and selectivity [3–6].

Bacterial contamination of platelets continues to be the single most significant cause of transfusion-associated morbidity and mortality [7–9]. Gram-positive bacteria (e.g., *Staphylococcus epidermidis* and *Staphylococcus aureus*) from skin are the most frequent contaminants in platelet units, whereas contamination of platelets with Gram-negative bacteria (e.g., *Escherichia coli*), while important, is less common [10]. A wide range of bacteria can proliferate quickly in platelet products, reaching clinically dangerous levels during storage at room temperature (RT). The overall rate of bacterial contamination has been estimated at about one per 1000–3000 units, causing reported clinical sepsis after about one per 20,000 transfusions [7]. The low bacterial load of contaminating bacteria in platelet products at the time of collection limits currently used detection methods. For example, several detection and inactivation

technologies [11–13] are currently used to reduce the risk of transfusion-transmitted sepsis; however, units containing small but clinically significant numbers of bacteria are missed [7,8,14].

Selecting aptamers that bind whole bacterial cells has an advantage in that multiple aptamers may potentially bind to different targets on the same cells in their native conformation and physiological environment, without requiring target protein isolation or purification. Aptamers can be selected without knowing the cellular target. A method called Systematic Evolution of Ligands by Exponential (SELEX) [15] has been used to select ssDNA aptamers that bind to bacteria of different species [16,17]. For example, whole-cell SELEX was conducted to select and characterize aptamers binding to *Salmonella typhimurium*, *E. coli* and *S. aureus* [3,18–20]. The combined use of multiple aptamers increased sensitivity for detection of whole *S. aureus* cells [20]. In addition to its use in diagnosis, a therapeutic aptamer binding an envelope protein of the hepatitis C virus was identified by whole-cell SELEX [21]. A therapeutic aptamer targeting the A1 domain of von Willebrand factor (vWF), a glycoprotein involved in blood coagulation, was used to inhibit vWF-mediated platelet activation, aggregation and thrombus formation [22–24]. The US FDA has approved one aptamer-based drug for marketing, pegaptanib sodium (Macugen®; an RNA aptamer directed against VEGF-165), and several others are currently undergoing clinical trials [1,25,26].

We investigated the potential use of aptamers to reduce the risk of platelet transfusion-transmitted infections. The aim of this study was to identify aptamers that bind specifically to the common bacterial pathogen *S. epidermidis* in platelet concentrates (PC). Previous studies developed several methods that rapidly identified and selected useful aptamers [27–29]. ►

► Aptamers with affinity to bacteria could be used to adsorb and remove bacteria contaminating PC, leaving the final product safe for transfusion. We envision this 'cleaning' step, possibly conducted just prior to platelet transfusion, to remove residual bacteria. To identify specific aptamers binding *S. epidermidis*, we applied the whole-cell-based rapid isolation of DNA aptamers (RIDA) method [30]. RIDA is an *in vitro* rapid one-step method to isolate high-affinity and high-selectivity aptamers without iterative cycles and amplification steps; it can be completed in 1 or 2 days, compared with conventional SELEX enrichment, which requires months and numerous rounds of selection (typically more than ten) to eliminate the large number of nonspecific DNA aptamers collected along with those of high affinity and specificity [6,31]. Using RIDA, we obtained a pool of DNA aptamers that went through selection and counter-selection steps from an aptamer library without requiring any amplification steps.

METHODS

Bacterial strains & culture conditions

S. aureus subsp. *aureus* Rosenbach (ATCC 27217), *S. epidermidis* (Winslow and Winslow) Evans (ATCC 49134) were grown in 10 ml of Tryptic Soy Broth at RT for 18 h with constant shaking (220 rpm). After incubation, bacterial cultures were diluted with media to a final optical density (OD) of 1.0 at 600 nm (approximately 1.5×10^9 cells/ml).

Aptamer isolation by rapid isolation of DNA aptamers

The initial population of DNA oligonucleotides was purchased from Integrated DNA Technologies (IDT; IO, USA). We selected the aptamers from a DNA library of custom-synthesized 76-mer ssDNA containing two flanking primer-binding regions and a random middle segment (34N). The library oligonucleotide sequence was 5'-TACGACTCACTATAGGGATCC-N34-GAATTCCTT-

TAGTGAGGGT-3' (N denotes number of randomly varied nucleotides). We screened the library of oligonucleotides against *S. epidermidis* using RIDA. Briefly, we filtered approximately 10^9 *S. epidermidis* cells using a sterile syringe and a sterile Millipore 0.45- μ m filter. Next, we washed with PBS the filter containing the trapped bacterial cells at a constant flow rate of 1.0 ml/min for 60 min. In the following step, we added 0.1 ml of an ssDNA library containing approximately 10^{15} randomized-sequence oligonucleotides (1.8 nM). Aptamers with affinity to *S. epidermidis* bound to the immobilized bacteria on the filter. We washed the filter with PBS, first at a flow rate of 0.05 ml/min and then at a flow rate of 1.0 ml/min for 24 h. For counter selection against plasma, we applied 100 ml of plasma (filter-sterilized through a 0.22- μ m filter) to the filter at a flow rate of 0.05 ml/min followed by washes with PBS for 30 min as described above. This step removed aptamers with strong binding affinity for plasma proteins. To remove *S. epidermidis* with bound aptamers, we opened the filter and transferred the bacteria to a tube with 1.0 ml PBS. The bacterial suspension was incubated with sodium thiocyanate (1.8 M) at RT for 30 min to elute surface-bound high-affinity nucleic acid ligands. We centrifuged the tube at $15,000 \times g$, collected the supernatant containing the aptamer candidates, and desalted the supernatant using a 3-kDa Centricon centrifugal filter (Millipore) with three washings of PBS. We mixed the final aptamer solution with 100 μ l of platelets. The platelets were fixed with formaldehyde and washed with PBS before incubating with final aptamer solution for 30 min at RT. The mixture was then centrifuged at $3000 \times g$ to collect the supernatant containing a pool of aptamer ligands with the highest affinity for *S. epidermidis*.

Cloning & sequencing

We amplified the ssDNA aptamer pool by

PCR (2 min at 95°C; 30 cycles of 95°C for 30 sec, 52.8°C for 30 sec, 72°C for 30 sec; final extension at 72°C for 5 min) from the pool of selected aptamers using the adapter region primers (Const forward and Const reverse, Table 1). We purified the PCR amplicon pool with a gel extraction kit (Qiagen, MD, USA) and cloned into the pCR2.1 TOPO vector using a TOPO TA cloning kit (Thermo Fisher Scientific, CA, USA). Next, we transformed the ligated vectors into One Shot® Top10 chemically competent *E. coli* cells (Invitrogen), plated transformed bacteria on LB agar plates with kanamycin (50 μ g/ml), and incubated at 37°C for 18 h. We propagated individual colonies of the transformed cells in 10 ml LB broth containing kanamycin (50 μ g/ml) overnight at 37°C, harvested the cells by centrifugation, and extracted plasmid DNA using the QIAprep® Spin plasmid Miniprep Kit. We screened colonies by PCR using M13 forward and M13 reverse primers and visualized the size of amplified products using 2% agarose gel electrophoresis. We selected transformants with an amplicon band size of 277 bp for sequencing. The empty vector generated a band of 201 bp. The DNA sequences of selected colonies were determined by Sanger sequencing (FDA, Facility for Biotechnology Resources, MD, USA).

Selection of aptamers based on estimates of free energy & secondary structure

We used the online software MFold (unafold.rna.albany.edu) to determine the folded secondary structures and thermodynamic properties ΔG (change in Gibbs free energy in units of kcal/mole) and melting temperatures (T_m) of these sequences. Next, we calculated the free energy of secondary-structure formation for each sequence by folding at 25°C using conventional ionic conditions (100 mM Na⁺, 2.25 mM Mg²⁺) used to store platelets [32] and other default settings. We selected ►

Table 1. DNA sequence of the primers used in the study.

Primer	Sequence
M13 forward (-20)	5'-GTAAACGACGGCCAG-3'
M13 reverse	5'-CAGGAAACAGCTATGAC-3'
Const region forward	5' TACGACTCACTATAG GGATCC 3'
Const region reverse	5' AACCTCACTAAAGGGAATT 3'

► aptamers based on the lowest ΔG values for the most stable secondary structures. The 5'-end biotin-labeled ssDNA of the selected aptamers and scrambled controls were generated (250 nmoles) by chemical synthesis and purified by ion-exchange high-performance liquid chromatography (IDT, IO, USA).

Binding assay

We used a direct colorimetric method to detect interactions between aptamers and the target bacteria. For the binding assay, we diluted *S. epidermidis* culture suspensions to 1.0 OD₆₀₀ (approximately 1.5×10^9 cells/ml). Briefly, we added 100 μ l of *S. epidermidis* cells ($\sim 10^8$) to 100 μ l of biotin-labeled SE40, SE42 and SE43 (2 μ M) or a control scrambled aptamer (2 μ M) into a 1.5-ml microcentrifuge tube and incubated the mixture at RT for 30 min to allow formation of the aptamer–bacteria complex. Next, we centrifuged the bacterial suspension at 13,000 $\times g$ for 5 min, discarded the supernatant containing unbound aptamers, and washed the pellet three times with PBS containing 1% BSA and 0.05% Tween 20 (wash buffer). We added a horseradish peroxidase (HRP)-conjugated streptavidin (ThermoFisher Scientific) at 1:16,000 in wash buffer and incubated for 30 min while rotating at RT. After washing three times with 1 ml of PBS by centrifugation, as described above, we resuspended the pellets in 50 μ l of PBS, added 150 μ l of 3,3',5,5'-tetramethylbenzidine solution (ThermoFisher Scientific) to the tubes, and incubated for 5 min at RT. The reaction was stopped by adding 150 μ l of stop solution containing 0.18 M sulfuric acid (ThermoFisher Scientific). The absorbance was read at 450 nm with sample volumes of 0.1 ml in 96-well microplates.

Binding assay for bacteria in platelets

We added 0.1 ml of bacterial suspensions (as described above) and aptamers in microcentrifuge tubes containing 0.4 ml of human PC. We immediately capped the microcentrifuge tubes and inverted them three times to mix, then incubated for 30 min while rotating at RT. Next, we centrifuged the tubes at 13,000 $\times g$ for 5 min and removed the unbound aptamers by washing three times with wash buffer. We added

Figure 1. Aptamer selection by rapid isolation of DNA aptamers and amplification of DNA aptamers from the aptamer pool. (A) Selection of aptamer binding with *S. epidermidis* with rapid isolation of DNA aptamers. (B) Agarose gel showing the PCR amplification of the aptamer pool isolated for binding *S. epidermidis*. Lane 1: DNA ladder; lanes 2 and 3: PCR product of 76-mer aptamer pool; lanes 4 and 5: control PCR with no template. (C) Colony PCR screening of 76-mer aptamers for *S. epidermidis*. We screened the selected transformed colonies using M13F and M13R primers. The migration of amplicons with plasmid containing aptamer (277 bp) versus empty plasmid (201 bp) is shown on the agarose gel. Lane 1: DNA ladder; lanes 2, 4, 5, 6 and 7: PCR showing colonies containing 76-nucleotide aptamers; lane 3: vector without insert.

HRP-conjugated streptavidin at 1:16,000 in wash buffer and incubated for 30 min while rotating at RT. The color reaction was developed as described.

RESULTS & DISCUSSION

This study describes a method for rapid single-step selection of ssDNA aptamers against surface sequences on *S. epider-* ►

Figure 2. Predicted folded structures of selected aptamers. 48 DNA aptamers with adapter regions were sequenced from a pool of aptamers isolated by binding with *Staphylococcus epidermidis*. Five aptamer sequences were selected based on lowest ΔG and highest melting temperatures. Secondary structures of selected aptamers were predicted using Mfold software; SE40 (A), SE42 (B), SE43 (C), SE48 (D) and SE52 (E). ΔG values represent the stability of the structure.

Figure 3. Colorimetric binding assay of biotin-labeled SE43 with *Staphylococcus epidermidis*. Biotin-labeled SE43 and scrambled control (2 and 4 µM) were incubated with $\sim 10^8$ *S. epidermidis* cells at room temperature for 30 min. The bacterial cells were washed with PBS containing 1% BSA and 0.05% Tween 20 (wash buffer), and incubated with streptavidin–horseradish peroxidase for 30 min. The bacterial cells with surface-bound aptamers were washed with PBS and subsequently incubated with 3,3',5,5'-tetramethylbenzidine as described in the methods. The data show the results from three independent assays with mean $\pm$ SD. *** p < 0.01 (p = 0.0035) for SE43 (2 µM) compared with no aptamer control and *** p < 0.01 (p = 0.0083) compared with scrambled control. * p = 0.0257 for SE43 (4 µM) compared with corresponding scrambled control, unpaired (two-tailed) t test with Welch's correction. OD: Optical density.

higher thermostability bind to bacteria better than those with lower stability, because higher stability generally correlates with higher binding affinity [33]. From this analysis, we identified five aptamers with lowest ΔG (highest thermostability) and the highest T_m : SE40, SE42, SE43, SE48 and SE52. We assessed their secondary structures using Mfold software (Figure 2, Table 2). Three of these five selected aptamers, SE40, SE42, SE43, and their respective scrambled controls, were further screened for binding interactions with *S. epidermidis* (10^8 cells) using 2 µM of each sequence in the biotin–streptavidin binding assay described above.

The selected aptamers and scrambled aptamers (Table 2) of the same nucleotide composition, but with randomly shuffled sequences, were synthesized with a biotin moiety at the 5'-end. We assayed the binding of these biotinylated aptamers to live *S. epidermidis* cells using colorimetric assays; the scrambled aptamers served as nonspecific binding controls. One aptamer, SE43, ►

► *midis* cells. Bacterial-cell-bound aptamers were separated using filters as described in the methods (Figure 1A). We performed a whole-cell RIDA against *S. epidermidis* with counter selection against plasma and platelets to enrich the aptamer pool with sequences specific for *S. epidermidis*. These two contra-selection steps were important to remove from the final pool those sequences having affinities to plasma proteins or to platelets (the matrix of concern). The aptamer pool contained 100 or more DNA sequences that we further screened with a selection process described below. Unlike conventional selection by SELEX, the method we applied produced a pool of aptamers that was successively screened to identify the best ligands. The new technique rapidly isolated aptamer-based ligands of high affinity for microbial pathogens without tedious isolation and purification of complex targets.

We amplified the eluted pool containing aptamers with the highest affinities to *S. epidermidis* using PCR with primers binding constant regions flanking the 34-nucleotide middle region (Figure 1B) and then selected 48 candidate aptamers to clone and sequence. The majority of these sequences showed single bands with PCR using M13F and M13R primers (Figure 1C). A few colonies

showed multiple bands of larger size (Figure 1C, lane 7) for an unknown reason. We did not sequence colonies yielding PCR products with multiple bands. In addition, we discarded those colonies that, upon DNA sequencing, revealed mismatched nucleotides in the flanking constant regions. All other sequences were used to characterize aptamers by identifying their secondary structures.

Next we ranked all sequenced aptamers based on computer-calculated stability of the secondary-structure folding. We further selected aptamers based on the assumption that aptamers with

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Table 2. Sequences and thermodynamic properties (ΔG and T_m) of 76-mer aptamers selected from a pool of aptamers isolated by rapid isolation of DNA aptamers.

Name	DNA sequence	ΔG (kcal/mol at 25°C)	T_m (°C)
SE40	TACGACTCACTATAGGGATCC ACCTATGGCAGATTGAGCCCAAGGGCTGTGCAGC GAATTCCTTTAGTGAGGGTT	-13.56	50.8
SE42	TACGACTCACTATAGGGATCC CCCCGAGTGAAGAGCAGGACAGCGGGACAGCGTC GAATTCCTTTAGTGAGGGTT	-12.51	52.9
SE43	TACGACTCACTATAGGGATCC GTGACTGTACGGGCTCAGTCGTTACTTGAGAGTT GAATTCCTTTAGTGAGGGTT	-13.56	52.8
SE48	TACGACTCACTATAGGGATCC AATGGCACAGCGCCTGGAACGTACTCTGTACCTG GAATTCCTTTAGTGAGGGTT	-14.12	51.4
SE52	TACGACTCACTATAGGGATCC TTCGCATCCGGCACGATGGCTAGGACACCCCGAT GAATTCCTTTAGTGAGGGTT	-12.75	53.1
Scrambled SE40	GCGCGATGGACTGTACCTTCGGCGTAACTGATATTCCTGATGATTCTGGCGAGACGTAGCACAAACCCG- GTGTATA	-7.19	42
Scrambled SE42	GTGCTTAGGTGCAGAACGCACACACGCTCGGTATGACGTGTTTCGTACGGTATAATTGCAGGCGCGAG- CACAAACAG	-11.77	52.6
Scrambled SE43	GGTATGTGTCGTTGATCTGGATATCTATGCCTAATGCTCGTAGGCGACTGTTCCGATAGTAACGTTGAC- CGCATAC	-5.89	43.6
Scrambled SE48	GTTGACGGATCCCTATTGCAGTGCTTGAGAGAGACCTCGCGTATATTTCCGAAGACCACGTGTAAT- GCCCTAACT	-2.69	39.7
Scrambled SE52	GTGCGTCGCATGATCGCGATTAGTGCACCATGTACGATTCTCACGACAACGACACTGTACTTGAG- GACTGTCTCC	-12.27	57.6

ΔG is the change in Gibbs free energy (in units of kcal/mol) and T_m is melting temperature. All these aptamers were selected for binding *Staphylococcus epidermidis*. Sequences of the aptamers are in bold.

► bound to *S. epidermidis* with at least a twofold increase in OD compared with those of the scrambled control and the background control (Figure 3). The OD did not increase as the amount of aptamer SE43 increased from 2 to 4 μM (Figure 3). We have no explanation for the lack of dose-response; it is likely due to variability from experiment to experiment.

In another study, SE40 and SE42 also showed higher binding relative to the background control; however, SE40 and SE42 showed no significant difference in OD compared with the scrambled control (Figure 4), indicating that these aptamers bound nonspecifically to bacterial cells. Figure 4 results confirm SE43 bound better than the corresponding scrambled aptamer.

We also characterized the aptamer binding with *S. epidermidis* cells spiked in PC. Human PC were mixed with a known final concentration (10^8 cells per assay) of

S. epidermidis to assess the performance of the candidate aptamer ligand in binding and capture of bacteria spiked in the relevant matrix. Our data showed that the optical density with aptamer SE43 bound to bacteria was four-times higher compared with scrambled control and background controls (Figure 5), indicating that aptamer SE43 bound well to *S. epidermidis*. We also detected twofold higher binding with SE43 in PC compared with the same assay conducted in PBS (data not shown). This result could be due to an optimized binding conformation of the aptamer in the ionic conditions of PC compared with PBS alone. The ODs were significantly lower when we tested platelets alone, aptamers alone (no bacteria) or bacteria alone (no aptamer) and were similar to the background signals. It is important to note that SE43 did not bind platelets (Figure 5), indicating that aptamer SE43 selectively

bound bacteria, even in a large excess of platelets. We believe that a negative-selection step successfully eliminated aptamers that bound nonspecifically to platelets and plasma. In preliminary studies, SE43 also showed a twofold increase in OD when tested with *S. aureus* in PBS and a fourfold increase when *S. aureus* bacteria were spiked in PC, compared with scrambled control and background control (data not shown). These data suggest that SE43 binding is not specific to *S. epidermidis*, or it could be interpreted that SE43 has a cross-species binding affinity useful to capture both strains of *Staphylococcus*.

FUTURE PERSPECTIVE

This work provides proof of principle that aptamers binding whole bacterial cells can be selected using a rapid one-step method, even without knowing the target binding sequence. Further development and full ►

Figure 4. Aptamers SE40 and SE42 do not bind with *Staphylococcus epidermidis* with high specificity. Biotin-labeled SE40, SE42 and SE43 and the scrambled controls (2 μ M) for respective aptamers were incubated with *S. epidermidis* cells at room temperature for 30 min. The cells were rinsed with wash buffer and incubated with streptavidin–horseradish peroxidase for 30 min. The aptamer-bound bacterial cells were washed with buffer and subsequently incubated with 3,3',5,5'-tetramethylbenzidine as described in the methods. Data are representative results from two independent tests with mean $\pm$ SD. OD: Optical density.

SUPPLEMENTARY DATA

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2018-0081.

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AUTHOR CONTRIBUTIONS

SJK and LG made substantial contributions to conception and design, and/or acquisition of data, and/or analysis and interpretation of data. VG and MD made substantial contributions to conducting assays, acquisition of data, analysis and interpretation of data. SJK, LG and DMA participated in conception of idea, design, drafting the article and revising it critically for scientific and intellectual content.

FINANCIAL & COMPETING INTERESTS DISCLOSURE

This work was funded by the US FDA. The RIDA method was conducted at Vivonics under a contract from the FDA. VG and MD were employed at Vivonics and conducted RIDA. VG shares in the RIDA patent. The FDA does not endorse any specific aptamer screening method. SJK was a fellow of the Oak Ridge Institute for Science and Education while at the FDA. Our comments are an informal communication and represent our own best judgment. These comments do not bind or obligate the FDA. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

ETHICAL CONDUCT OF RESEARCH

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all

► characterization of *S. epidermidis*-specific aptamers, including identification of the dissociation constant, requires confirmation by other methods such as qPCR and flow cytometry analysis (target bacteria labeled with increasing concentrations of aptamer candidate) in future investigations. In addition, we will test the

function of platelets exposed to SE43 aptamer [10] and evaluate its binding to other bacterial species. The final goal is to incorporate this aptamer (and others if needed) into filtration devices to capture and remove small numbers of bacteria that may contaminate platelets.

Figure 5. Binding assay of SE43 with *Staphylococcus epidermidis* spiked in human platelet concentrates (PC). Biotin-labeled SE43 (2 μ M) was mixed with *S. epidermidis* in PC diluted in PBS and incubated at room temperature for 30 min. The bacterial cells were washed and incubated with streptavidin–horseradish peroxidase for 30 min. The aptamer/bacterial cell complex was washed with wash buffer and subsequently incubated with 3,3',5,5'-tetramethylbenzidine as described in the methods. The background control samples included bacteria incubated in PC, aptamers incubated in PC, and platelets alone. The data shown are results from three independent tests with mean $\pm$ SD. **** p < 0.0001 compared with corresponding scrambled control and *** p = 0.0001 compared with no aptamer control, unpaired (two-tailed) t test with Welch's correction. OD: Optical density.

► human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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