We have developed a selection scheme to generate nucleic acid sequences that recognize and directly internalize into mammalian cells without the aid of conventional delivery methods. To demonstrate the generality of the technology, two independent selections with different starting pools were performed against distinct target cells. Each selection yielded a single highly functional sequence, both of which folded into a common core structure. This internalization signal can be adapted for use as a general purpose reagent for transfection into a wide variety of cell types including primary cells.

INTRODUCTION

RNA molecules can be internalized into cells and used to deliver reporters and therapeutic cargoes.1–6 Aptamers that bind to cell surface markers such as the cancer marker prostate-specific membrane antigen (PSMA),7 epidermal growth factor receptor (EGFR)8 or the viral protein gp1209 appear to deliver their cargoes via endocytosis.10–13 These findings are particularly significant given that aptamers can be readily synthesized, facilitating their site-specific conjugation to a variety of reporters or therapeutic moieties,14,15 a feat that is still difficult for antibodies and many other protein-based targeting agents.

The relationship between aptamer binding, uptake, and functional delivery appears to be idiosyncratic. Because the mechanism is still poorly understood, we reasoned that a selection approach would be useful both for generating superior internalizing reagents and also for providing additional insights into the routes by which aptamers deliver cargoes. Selections against whole cells have previously been carried out,16–22 and some have yielded aptamers capable of being internalized by cells.22 We have now augmented this technology to enable the selection of nucleic acids that could not only recognize a cell, but also enter it. Briefly, a nucleic acid pool was added directly to cells, and nucleic acids that failed to internalize were removed by stringent nuclease treatment. Total cell RNA was extracted, and internalized sequences were recovered by reverse transcription-PCR and transcription (Figure 1a). Surprisingly, when we performed selections using two different libraries against cell lines from two different species, we identified a common core motif that appears to be a general internalization signal for RNA. Much like the cell surface selections, it should be possible to generalize this method to many different cells and tissues, even in the absence of any knowledge of mechanism or cell surface architecture.

RESULTS

Proofing the selection method

Selections were initiated with RNA pools containing 2′-ribo purine/2′-fluoro pyrimidine (2′-fluoro-modified). This modification renders RNA largely nuclease-resistant.21 We initially proofed the method with a known internalizing aptamer, the anti-PSMA aptamer, A97,10,15 (Figure 1b). Approximately 10^6 PSMA-expressing LnCAP cells were treated with one of four conditions:

1. Cells were incubated with the anti-PSMA aptamer.
2. Cells were initially treated for 10 minutes with sodium azide (Az), an inhibitor of oxidative phosphorylation, and 2-deoxyglucose (dG), an inhibitor of glycolysis, to prevent endocytosis before addition of the aptamer.
3. Cells were incubated with the anti-PSMA aptamer followed by treatment with nuclease to digest external binders. While the 2′-fluoro modifications rendered the RNAs largely resistant to cellular nucleases, we had previously identified conditions that would degrade a modified RNA pool. RNase T1 (which cleaves after G residues) and RNase A (which cleaves single-stranded RNA (ssRNA)) treatment led to only partial degradation of the pool, but Riboshredder (Rb), a commercial cocktail of nucleases, effectively eliminated all full-length pool RNAs (data not shown).
4. Cells were pre-treated as in (1), then incubated with the aptamer RNA and, finally, nuclease-treated as in (3) to remove surface binders.

Following treatment, total cell RNA was recovered from each sample, reverse-transcribed with aptamer-specific primers, and the number of aptamers recovered was determined relative to a nontreated cell control. As might be expected, treatment with Az-dG decreased the total amount of recovered RNA. Treatment

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with Riboshredder reduced the signal even more drastically, indicating that much of the aptamer remained extracellular. However, some signal (approximately twofold over background) remained, presumably due to internalization (Figure 1b; Supplementary Figure S1). Treatment of the cells with both Az-dG and Rb brought the amount of RNA detected down to background levels. A similar experiment with another internalizing RNA, the anti-EGFR aptamer, E07, also showed that endocytosis inhibition led to a decrease in the amount of RNA recovered following nuclease treatment. In this case, the RNA was fluorescently labeled with phycoerythrin and analyzed by flow cytometry (Figure 1c) rather than being subjected to reverse transcription-PCR. As observed with the anti-PSMA aptamer and anti-EGFR aptamer, surface bound RNA could be destroyed by treatment with Riboshredder leading to a decrease in the observed fluorescence. The congruence between the two aptamers and the two methods strongly suggested that we could recover internalized RNA in the context of a selection.

Internalization selections

A test selection was initially carried out using a partially randomized (“doped”) library composed of ~10^13 unique variants based on the anti-PSMA aptamer, A9. Three rounds of internalization selection were carried out against HeLa cells stably expressing PSMA (HeLa-PSMA) on their surfaces. In each round, the doped library was added directly to cells in media and incubated for 1 hour, after which the cells were washed several times, and nucleic acids that failed to internalize were further removed by nuclease treatment. Following this step, total cell RNA was extracted, and the internalized sequences were recovered by reverse transcription-PCR and subsequently transcribed back into RNA. While the Round 0 library lacked the ability to label cells (as demonstrated by flow cytometry; Figure 1d), successive rounds of selection resulted in increased levels of binding with the Round 3 population labeling cells as well as the wild-type A9 aptamer. Further analysis of clones from Round 3 showed that 14 out of 19 variants bound PSMA-positive cells as well as or better than the parental aptamer (Supplementary Figures S2, S3, and S4).

Having shown that we could select for the internalization of a receptor-binding aptamer, we then wished to select for internalization of sequences without regard to receptor or mechanism. We initiated two different internalization selections: one in which we targeted the common human cancer line, HeLa, and another in which we targeted the murine, glucose-responsive, immortalized β-cell line, Min6B1.

For the selection against HeLa cells, we used a 2′-fluoro-modified RNA aptamer pool that contained 62 random sequence positions and spanned ~10^14 unique sequences. For the first round, we incubated the RNA pool with 5 × 10^6 cells in complete cell culture media for 30 minutes at 37 °C. Following incubation,
cells were washed three times with Dulbecco’s phosphate-buffered saline (DPBS), and both surface-bound and internalized RNAs were isolated by Trizol extraction. The recovered RNA was reverse-transcribed, PCR-amplified, and then transcribed for use in subsequent rounds. Following Round 2, the selection stringency was increased by trying to keep cell surface proteins and by digesting surface-bound aptamers with Riboshredder before recovery and amplification of internalized aptamers. The overall selection was carried out for a total of six rounds.

Analysis of individual rounds from the selection by flow cytometry revealed a marked increase in the fitness of the library after five rounds of selection and amplification. Flow cytometry assays conducted without (Figure 2a) or with Riboshredder treatment (Figure 2b) suggested that a significant portion of the fluorescently-labeled pool was being internalized by the cells.

Selections targeting the immortalized mouse β-cell line, Min6B1, were performed similarly to those described above for HeLa cells. For this selection, a 2′-fluoro-modified RNA library encompassing 50 random positions was used as a starting point. Starting with ~10^14 unique sequences, six initial rounds of selection were performed in which the library was incubated with cells for 1 hour in full media. The cells were then washed stringently to remove any non-bound sequences, and cells were lifted from the plate by treatment with trypsin and ethylenediaminetetraacetic acid (EDTA), but no nuclease. For Rounds 7 through 10, a nuclease step was added following trypsinization. For Rounds 11 and 12, freshly isolated mouse islets (which are composed of ~80–90% primary β-cells) were used as target cells instead of cultured Min6B1.

Flow cytometry performed with individual rounds from this selection indicated a progressive increase in the ability of the populations to bind Min6B1 cells, with Rounds 11 and 12 displaying the highest signal (Figure 2c). Interestingly, Round 7, which was the first round to receive a nuclease treatment step, showed a marked shift in the cytometry profile, suggesting that internalization greatly improved recovery.

Identification of an internalization motif

Sequence analysis of the Round 6 population selected against HeLa cells yielded a single, dominant clone (dubbed “Otter”) which comprised 48% (15/31) of the population. A second clone comprised an additional 26% (8/31) of the population. Two other clones appeared twice, and the rest of the internalizing sequences appeared only once (Supplementary Figure S5). Flow cytometric analysis of the individual clones revealed that Otter seemed to internalize readily, and, to a lesser extent, so did the “runner up” (Figure 2b; data not shown). As a control for surface binding, Otter was exposed to the same Riboshredder concentrations used in the selection experiment and was found to be completely degraded after 10 minutes of exposure to the RNase cocktail (Supplementary Figure S6).

Similarly, sequence analysis of 17 clones from the Round 12 population selected against Min6B1 cells revealed one dominant sequence (clone 1; c1) which encompassed 50% (8/17) of the population (Supplementary Figure S7). We assayed this as well as nine other unique sequences from the Round 12 population by flow cytometry. In this instance, only the dominant clone, c1, showed a significant (~100-fold) signal increase (data not shown).

A striking feature of the selections against HeLa and Min6B1 cells was the apparent “takeover” by a single functional sequence. This was far from a given; in at least one reported selection against whole cells, the dominant sequence (which encompassed ~50% of the population at the end of the selection) turned out to be non-functional.17 Surprisingly, a comparison of the dominant sequences from both selections revealed two similar regions: a pyrimidine rich region (Figure 2d; green) and the sequence 5′ CUUUAUGAU (Figure 2d; red).

Generality of internalizing RNAs

The fact that a common internal motif was discovered for RNAs selected on two different cell lines suggested that the mechanism of internalization might be common to many different cell lines, and that therefore, the aptamers might be internalized by many different cell lines. In this regard, by Round 5, the aptamer population selected against HeLa cells was also found to be taken up by A431 and other cells (Supplementary Figure S8), albeit with slightly different efficiencies. For example, the ratio of bound to internalized aptamer on A431 cells was ~1:1 while the ratio on HeLa cells was 1:0.89 (Supplementary Figure S9). Differences in...
RNA internalization appeared to be both cell type- and aptamer-specific, as the anti-EGFR aptamer E07 showed a reversed trend of uptake efficiency (1:0.22 for A431 cells and 1:0.52 for HeLa cells).

Similarly, we found that c1 was capable of staining not only Min6B1 cells, but other mouse lines including BTC3, Bend.3 (Supplementary Figure S10), Sol8, and C2C12 cells (data not shown) as well as a number of human cell lines including Jurkat, A549, HEK293, HeLa, PC3 (Supplementary Figure S10), and LnCAP cells (data not shown).

Based on previous results with other aptamers, it seemed likely that the common mechanism of internalization might be endocytosis. When Min6B1, Bend.3, and HeLa cells were treated with fluorescently-labeled c1 for 1 hour and then imaged by microscopy, punctate staining was observed, supporting the notion of uptake and localization of the internalizing sequences within endosomes (Figure 3a). To investigate the mechanism of uptake further, we performed flow cytometry experiments with Jurkat cells in the presence of either phenylarsine oxide, an inhibitor of clathrin-mediated endocytosis,24–28 or indomethacin, an inhibitor of caveolin-mediated endocytosis27,28 (Figure 3b). Treatment with phenylarsine oxide resulted in only a slight decrease in overall fluorescent staining (Figure 3b; top, orange) that could be reduced to near background levels following treatment with RNase (Figure 3b; top, blue). Treatment with indomethacin had little or no effect on binding or observed uptake (Figure 3b; bottom). Similar results were observed when the same experiment was performed on HEK EBNA cells (Supplementary Figure S11). Taken together, these experiments suggest that upon receptor binding, c1 is internalized into mammalian cells by a clathrin-mediated endocytosis.

Minimization and exploitation of the common “internalization motif”

Starting from c1, we generated a series of truncations in which we progressively deleted both the 3′ and 5′ portions of the sequence (Supplementary Figure S12). The minimized constructs were initially synthesized enzymatically bearing a common 3′ constant region which was used for hybridization of a biotinylated oligonucleotide and labeled with phycoerythrin-labeled streptavidin. Constructs were then assayed for internalization by flow cytometry (data not shown). A minimized variant, c1.min, that was 41 nucleotides in length was chemically synthesized bearing a 5′ biotin and a 3′ inverted dT residue to enhance serum stability (Figure 4a; c1.min). This variant retained full activity (Figure 4b). Comparison of the predicted structure of c1.min with that of full-length Otter showed that these aptamers indeed shared the predicted common motif and structural core (Figure 4a). To prove that these shared motifs were the basis of function, we used c1.min as a model and designed a rational truncate of Otter, maintaining the similar core domains (Figure 4a; Otter.min). The minimized Otter still bound and was internalized into cells (Figure 4b). To confirm that these molecules interacted with the same molecular target on the cell surface, we also performed competition experiments for binding to Jurkat cells using an AF647-labeled Otter (Otter-AF647) and fluorescein isothiocyanate (FITC)-labeled 1 (c1-FITC) by flow cytometry. With the concentration of Otter-AF647 kept constant, increasing concentrations of c1-FITC resulted in an increase in the observed FITC signal (Figure 4c) and a concomitant decrease in the AF647 signal (Figure 4d) demonstrating that these aptamers compete for binding. Competition was further confirmed by microscopy where both Otter and c1 were found to co-localize within the same cellular compartments (Supplementary Figure S13).

The facile synthesis of a small nuclease stabilized RNA which is readily taken up by many different cells paves the way for the development and enhancement of novel RNA-based transfection strategies. Using the minimized c1, we performed an additional set
of staining experiments using normal human primary bronchial epithelial cells (NHBECs). Similar experiments were performed in parallel using two immortalized lung cell lines: A549 cells, an adenocarcinoma of human alveolar basal epithelial cells, and HBEC, human bronchial epithelial cells immortalized by overexpression of telomerase. As shown in Figure 5, the minimized constructs bound to and were internalized by NHBECs to an extent similar to that observed for the transformed lines. Internalization into the primary NHBECs was further confirmed by treatment with endocytosis inhibitors as described above (Supplementary Figure S14).

To extend our observations for generalized c1 uptake to a wider range of cell lines and to primary cells in vivo, we used an established model of intravaginal delivery to assess aptamer uptake in cells of the genital mucosa. Fluorescently-labeled aptamer c1, control aptamer (c36) or phosphate-buffered saline (PBS) (150 pmol each) were administered vaginally to mice. Analysis of vaginal tissue isolated 24 hours after aptamer application showed fluorescence deep into the lamina propria only following application of c1 (Figure 6a,b). No fluorescence was observed in tissues treated with c36 or PBS (Figure 6c,d).

**DISCUSSION**

The need for a facile, robust, and effective means of delivering drugs or other molecular cargoes into cells led us to design a selection scheme for RNA molecules that could internalize into cells independent of transfection reagents or physical manipulation. Interestingly, our selections against two very different cell lines, one a human cancer line and the other a mouse immortalized islet cell line, yielded internalizing RNAs with strikingly similar sequential and structural properties. By combining the core motif with different cargoes and modifications, we may have generated a universal nucleic acid delivery reagent, similar to the Tat peptide that has been used in hundreds of scientific studies as well as in clinical trials. When the aptamer was applied to the vaginal mucosa of mice the observed staining and uptake patterns were similar to those seen following the administration of small interfering RNAs (siRNAs) using transfection agents or following the administration of cholesterol-derivatized siRNAs.

Just as the mechanism of uptake for Tat has been slowly elucidated, it seems that c1 can be readily utilized by researchers even as the pathway of internalization is further explored. Thus
MATERIALS AND METHODS

Cell culture. A431, Bend3, and HeLa cells were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco's modified Eagle medium (DMEM) (ATCC) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA). LnCAP cells were also purchased from ATCC and were cultured in RPMI-1640 (Invitrogen) supplemented with 10% FBS.

Min681 cells were a kind gift of Dr Jeffrey Pessin at the Diabetes Center at the Albert Einstein College of Medicine, New York, and were cultured in DMEM supplemented with 10% FBS. HBEC, A549, and primary NHBE cells were a kind gift of Dr Simon Spivack at the Albert Einstein College of Medicine, New York and were cultured in F12 Kainhn's medium supplemented with 10% FBS. HeLa-PSMAs cells were generated in our lab using a PSMA expression vector in which the open reading frame (positions −128 to 2,253) was inserted between the KpnI and XbaI of the EF1 promoter driven vector pEF6-V5HisA (Invitrogen).

All cells were grown at 37 °C with 5% CO2 and 99% humidity.

Aptamer libraries, aptamers, and oligonucleotides. All primers used for PCR as well the biotinylated oligonucleotides used for flow cytometry were purchased from IDT (Coralville, IA). All pools and minimized aptamers were synthesized in our lab on an Expedite 8909 DNA synthesizer (Applied Biosystems, Carlsbad, CA) using standard synthesis procedures. Unless noted otherwise, all reagents were purchased from Glen Research (Sterling, VA).

Libraries were synthesized such that N regions contained an equal probability of containing A, T, G, or C, as previously described.34 Following deprotection, libraries were gel purified by denaturing (7 mol/l urea) gel electrophoresis on an 8% polyacrylamide gel. The single-stranded DNA library was amplified by PCR to generate a double-stranded DNA bearing a T7 promoter and transcribed in vitro using the Y639F mutant of T7 RNA polymerase35,36 or Durascribe kits and 2'-fluoro-pyrimidines. The RNA was purified on a denaturing (7 mol/l urea) 8% polyacrylamide gel.

Biotinylated, minimized aptamer variants were chemically synthesized in our lab using 2'-fluoro-dC and 2'-fluoro-dU phosphoramidites (Metkinen, Kuusisto, Finland) bearing an inverted dT residue for added stability. Thiolated, minimized aptamer variants were synthesized using a thiol-modifier C6 S-S phosphoramidite. All aptamers were synthesized with 4,4'-dimethoxytrityl on. Following deprotection, aptamers were purified by reversed phase high-performance liquid chromatography (HPLC) on a 10 × 50 mm Xbridge C18 column (Waters, Milford, MA) using a linear gradient of acetonitrile in 0.1 mol/l triethylammonium acetate at pH 7.0.

Thiolated aptamers were used to generate the AlexaFluor488 (A488)-labeled aptamer used for in vivo microscopy studies. Labeling was performed using AF488-C5-maleimide as follows: 10 nmol of thioltated aptamer was reduced using 10 mmol/l tricarboxyethylphosphine in 100 μl of 0.1 mol/l triethylammonium acetate. Samples were heated at 70 °C for 3 minutes followed by incubation at room temperature for 1 hour. The reduced aptamers were desalted using a Biospin 6 column (Bio-Rad, Hercules, CA) into PBS supplemented with 50 mmol/l phosphate, pH 7.5. To this, 10 μl of dimethyl sulfoxide (DMSO) was added containing 50 nmol AF488-C5-maleimide. Following an overnight reaction at 4 °C, the oligonucleotide was recovered by ethanol precipitation and desalted an additional time using a Biospin 6 column. Dye to aptamer ratios were determined at 260 and 488 nm and were typically ~1. The absence of free dye in the final product was confirmed by reversed phase HPLC.

Real-time PCR analysis of aptamer binding and internalization. The anti-PSMA aptamer, A9, was used to proof internalization into cells. Approximately 50,000 PSMA-positive LnCAP cells were plated per well in a 24-well plate and allowed to grow overnight such that cells would be at ~105 cells/well at assay time. The cells were washed in DPBS (Invitrogen) and treated with one of four conditions: 50 nmol/l anti-PSMA aptamer was added directly to the media on cells (RNA); cells were first treated for 10 minutes with 10 mmol/l...
Az and 50 mmol/l dG to prevent endocytosis, then RNA was added to the media after washing (Az-dG); RNA was added to cells, and then cells were nuclease-treated with 0.02 U/μl Riboshredder (Epicentre, Madison, WI) to digest species bound to the cell surface (Rb); or cells were treated with Az-dG, washed and treated with RNA and nuclease-treated (Az-dG/Rb). Each treatment was performed in triplicate. Total cell RNA from each of the treatments was extracted with Trizol (Invitrogen) following manufacturer’s protocols. For reverse transcription, 500 ng total cell RNA was incubated with 5 pmol aptamer-specific reverse primer (5′-TCGGGCGAGTCGTCTG-3′), 5 pmol pool-specific primer (5′-AAGCTTCGTCAAGTCTGCAGTGAA-3′), and 5 pmol glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reverse primer (5′-GAAGGTGAAGGTCGGAGT-3′) in First Strand Buffer. The solution was heated to 70 °C for 3 minutes and then cooled on ice. A solution of 50 nmol dithiothreitol, 5 pmol each deoxynucleotide triphosphate and 50 U Superscript III reverse transcriptase (Invitrogen) was added to the primer mix for a final total volume of 10 μl and incubated at 50 °C for 1 hour followed by an inactivation step at 70 °C for 15 minutes.

Real-time PCR was performed using a 7300 Real Time PCR System (Applied Biosystems). PCR reactions consisted of 1 μl of reverse transcription reaction, 12.5 μl SYBR Green Mastermix (Applied Biosystems), 10 pmol target-specific reverse primer, and 10 pmol target-specific forward primer in a total volume of 25 μl. Separate reactions were performed to amplify the aptamer (forward primer: 5′-TTCTAATAACGACTCACTATAAGGGAGAACGGATCGCG-3′), GAPDH (forward primer: 5′-TGAGTCGTGTCGCTGTCTG-3′). Cycling conditions started with an initial denaturation step at 95 °C for 10 minutes followed by 40 cycles of 95 °C for 30 seconds, 50°C for 30 seconds, and 72°C for 1 minute. A final cycle of 95°C for 15 seconds, 60°C for 30 seconds, and 95°C for 15 seconds was used to generate dissociation curves to check the integrity of the products. The levels of each species were calculated by the ΔΔCt method by taking the difference in Ct between each sample and cells control and then normalizing to GAPDH levels for each sample.

Doped-A9 selection. The sequence of the doped-A9 library used for the selection was, 5′-GGGAGGACGATGCAGGACCGAAAAGACCTGACTTCTATCAGTATACGGAGACGACTCGCCCGATTGAA TTAAATGCCCCCATCACCAG-3′, where the underlined residues were doped to a level of 30%. Library preparation was performed as described above.

The internalization selection was performed using HeLa-PSMA cells. For each round, ~10^7 adherent cells were incubated in 300 μl of DMEM supplemented with 10% FBS and 1 mg/ml transfer RNA (tRNA) and ssDNA (Sigma, St Louis, MO) as blocking agents. The first round of selection used 30 μg of the A9 doped pool encompassing ~10^6 unique variants. Subsequent rounds were performed using 3 μg of RNA. For each round of selection, the pool was combined with a 1.5-fold molar excess of reverse primer bearing a 5′ fluorescein dye. The mixture was denatured at 70 °C for 3 minutes and allowed to refold at room temperature for at least 15 minutes before addition to cells. RNA was added to the blocked cells and incubated for 1 hour at 37°C. Following incubation, the cells were washed three times with 1 ml HBSS (Hank's buffered saline solution; Invitrogen) containing 0.1% Az, once with 1 ml cold 200 mmol/l glycine and 150 mmol/l NaCl at pH 4, and three more times with HBSS. Cells were lifted with 500 μl trypsin-EDTA (Mediatech, Manassas, VA) containing 0.1% Az, removed from the plate, washed with 1 ml HBSS and resuspended in 100 μl HBSS containing 5 μl Riboshredder RNase cocktail (Epicentre). Following a 15 minute incubation at room temperature, the cells were washed an additional three times with 1 ml of HBSS. Cells were lysed, and RNA was recovered using Trizol extraction according to manufacturer’s protocol. Recovered RNA was reverse-transcribed and amplified by PCR. Double-stranded DNA was ethanol precipitated and transcribed back into RNA. The progress of the selection was monitored by flow cytometry as described below.
Following the third round of selection, the pool was cloned using the TOPO TA kit (Invitrogen) and 19 clones were sequenced, transcribed, and assayed by flow cytometry for their ability to bind to wild-type HeLa cells or HeLa-PSMA cells.

**HeLa cell internalization selection.** The sequence of the N62 library used for selection against HeLa cells was: 5′-GGCGCTCCGACCTTATGTCCTG-N₆₆₋₆-GAACCGTTGAGACACCAG-G-3′. Library preparation was performed as described above. Each round of selection employed 1 nmol RNA, which corresponds to a starting library size of ~10¹⁴. Thermally equilibrated RNA was added to 5 ml of media in a T-75 flask containing ~6 × 10⁶ HeLa cells and incubated for 30 minutes at 37°C with 5% CO₂. Cells were then washed with 10 ml DPBS three times and lifted with trypsin. The cells were suspended in 5 ml of DMEM supplemented with 10% FBS and recovered by centrifugation at 200g for 5 minutes. The cell pellets were each resuspended in 100μl DPBS, combined, pelleted, and washed three more times in 1 ml DPBS. Total cell RNA was recovered using the mirVana Kit (Invitrogen) according to manufacturer's protocol, and eluted in 40μl of elution buffer; 20μl of the isolated RNA was used in a 40μl reverse transcription reaction using SuperScript III reverse transcriptase (Invitrogen) and the N62 reverse primer according to manufacturer's protocol. Following PCR amplification, the double-stranded DNA was ethanol precipitated, transcribed back into RNA and purified as described above. After Round 2, the cells were washed four times as described above, then resuspended in 100μl DPBS containing 5 mmol/l MgCl₂ and 10μl Riboshredder RNase. The reaction was allowed to proceed for 15 minutes at room temperature after which the cells were washed five times in 1 ml DPBS and resuspended in 100μl DPBS supplemented with 10μl of RNase Out (Invitrogen). The mixture was left at room temperature for 2 minutes after which the cells were pelleted, and the RNA was isolated as described above.

**Min6B1 cell internalization selection.** The sequence of the N50 library used for selection against Min6B1 cells was: 5′-GGCGCTCCGAGGAATGGTTCTACGAT-N₆₆₋₆-TTACATGGGAGTGACACCAGTAAATGATTTGCGGCGG-CATGACCA-G-3′. Library preparation was performed as described above. For the first round of selection, Min6B1 cells were grown as an adherent monolayer and cultured in a 6-well plate to about 90% confluence (~10⁶ cells). Cells were blocked with 0.1μg/ml trRNA and ssDNA in 1 ml DMEM supplemented with 10% FBS for 30 minutes at 37°C before the addition of 1.2nmol of thermally equilibrated RNA library (~10⁶ sequences). Following a 1 hour incubation at 37°C, the cells were washed three times with 1 ml HBSS to remove unbound RNA, and then cells were lifted by the addition of trypsin in the presence of 10mmol/l EDTA for 20 minutes at 37°C. Cells were recovered by centrifugation and washed three times with 1 ml HBSS. Total cell RNA was Trizol extracted, according to the manufacturer's protocol. Recovered RNA was reverse-transcribed using MMLV reverse transcriptase (Invitrogen) and the N50 reverse primer according to the manufacturer's protocol. Following PCR amplification, the double-stranded DNA was ethanol precipitated and transcribed back into RNA and purified as described above. For Rounds 7–10, an RNase treatment step followed trypsinization. Cells were incubated in 500μl of HBSS containing 0.1% azide and 5μl of Riboshredder RNase. Cells were then washed three times with 1 ml HBSS and fixed with 4% formaldehyde solution for 20 minutes at room temperature. The fixed cells were washed three times with PBS and mounted in mounting media (0.1 mol/l propylgalact, 50% glycerol in PBS) containing 1 μg/ml 4'-6-diamidino-2-phenylindole (DAPI). Cells were also assayed without fixation and visualized on a Zeiss Axio Observer fluorescence microscope (Thornwood, NY).

**Microscopy.** For microscopic analysis of Min6B1, HeLa, and Bend3 cells, cells were grown as adherent monolayers in Labtek 8-chamber glass slides systems (Thermo Scientific, Rochester, NY). Thirty minutes before aptamer treatment, cells were blocked with 0.1μg/ml trRNA and ssDNA in DMEM supplemented with 10% FBS at 37°C. The blocked cells were then incubated with 100nmol/l 5′-biotinylated c1 or 5′-biotinylated control aptamer conjugated to streptavidin-R-phycocerythin (Prozyme) at 37°C for 1 hour. Cells were then washed three times with PBS and fixed with 4% formaldehyde solution for 15 minutes at room temperature. The fixed cells were washed three times with PBS and mounted in mounting media (0.1 mol/l propylgalact, 50% glycerol in PBS) containing 1 μg/ml 4'-6-diamidino-2-phenylindole (DAPI). Cells were also assayed without fixation and visualized on a Zeiss Axio Observer fluorescence microscope (Thornwood, NY).

**Preparation of adult mouse islets.** Pancreatic islets were isolated from 5- to 8-week old male BALB/c mice. The pancreas was first dissected by intraductal injection of collagenase solution (1 mg/ml in HBSS). Collagenase digestion was performed at 37°C for 15 minutes. Subsequently, the pancreas was excised, and the digestion was quenched by addition of cold HBSS. The islets suspension was washed three times with cold HBSS, and islets were separated by centrifugation on a Histopaque discontinuous gradient for 20 minutes at 1700g at 4°C. Islets were picked and counted under an inverted microscope. The islets were cultured in 100 mm dishes at 37°C in humidified 5% CO₂ in RPMI-1640 medium supplemented with 10% FBS and 1× penicillin/streptomycin solution (Invitrogen) for 5 days.

**Binding assay by flow cytometric analysis.** Aptamer binding and uptake was assessed by flow cytometry. Rounds from each selection or isolated clones were first hybridized to a biotinylated oligonucleotide (B-CTGGTCATGGCGGCATTTAATTC) which was complementary to the 3′ end of the library. The population or individual aptamer was incubated at 1μmol/l with 1.5μmol/l biotinylated oligonucleotide in DPBS heated to 70°C for 3 minutes and then allowed to cool on the bench top for 15 minutes. Following hybridization, the aptamers were complexed with 1.5μmol/l streptavidin-R-phycocerythin (Prozyme, Hayward, CA) for an additional 15 minutes. For assays performed with biotinylated minimized aptamers, following thermal equilibration, 1μmol/l aptamer was incubated with 1.5μmol/l streptavidin-R-phycocerythin and incubated for 15 minutes before addition to cells.

For the assay, cells were first blocked with 0.1μg/ml trRNA and ssDNA in DMEM supplemented with 10% FBS for 30 minutes at 37°C. Aptamer complexes were added to cells at a final concentration of 50–100nmol/l (as indicated) for 30–60 minutes (as indicated) at 37°C and 5% CO₂. Cells were then washed three times with DPBS, trypsinized and washed with fluorescence-activated cell sorting (FACS) buffer (HBSS, 1% bovine serum albumin, 0.1% Az). Samples designated to test internalization were treated with Riboshredder cocktail as described above for the selection. After incubation, cells were washed twice with FACS buffer and resuspended in FACS buffer with 7-aminoactinomycin D stain (7AAD) to exclude dead cells in the analysis. Flow cytometry was performed on a FACSScan or FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

**Competition binding assay by flow cytometry analysis.** Approximately 5 × 10⁶ Jurkat cells were blocked with 0.1μg/ml trRNA and ssDNA in DMEM supplemented with 10% FBS for 30 minutes at 37°C. Cells were then incubated with 400nmol/l 5′-biotinylated Otter aptamer conjugated to AlexaFluor-647-labeled streptavidin (Invitrogen) and increasing concentrations (0, 0.2, 0.4, 0.8, 2, and 4μmol/l) of either 5′-biotinylated c1 or 5′-biotinylated control aptamer conjugated to streptavidin-R-phycocerythin (Prozyme) at 37°C for 1 hour. Cells were then washed three times with PBS and fixed with 4% formaldehyde solution for 15 minutes at room temperature. The fixed cells were washed three times with PBS and mounted in mounting media (0.1 mol/l propylgalact, 50% glycerol in PBS) containing 1 μg/ml 4'-6-diamidino-2-phenylindole (DAPI). Cells were also assayed without fixation and visualized on a Zeiss Axio Observer fluorescence microscope (Thornwood, NY).

**Endocytosis inhibition.** Jurkat, HEK EBNA 293, or NHBE cells were incubated with either 10μmol/l phenylarsine oxide or 100μmol/l indomethacin in RPMI-1640 media at 37°C during 30 minutes. Cells were then blocked with RPMI-1640 medium supplemented with 10% FBS and 0.1μg/ml trRNA and ssDNA for 30 minutes at 37°C; 100nmol/l 5′-biotinylated c1 or 5′-biotinylated control aptamer conjugated to streptavidin-R-phycocerythin (Prozyme) was added to the media and incubated at 37°C for 1 hour. RNase treated samples were further incubated with 10μl of RNase Riboshredder (Epipcentre) in RPMI-1640 supplemented with 10% FBS. Cells were washed with HBSS and analyzed by flow cytometry.
**Vaginal uptake experiments.** C57BL/6 mice (Taconic, Hudson, NY) were subcutaneously injected with 2 mg medroxyprogesterone acetate (Greenstone, Peapack, NJ) to maintain them in diestrus. One week later, 150 pmol c1 aptamer or control c36 aptamer labeled with AlexaFluor A488 were administered intravaginally in 15 μl of DPBS. Twenty-four hours later, vaginal tissue was dissected, placed in Tissue-Tek optimal cutting temperature compound (Sakura Finetek, Torrance, CA) and snap-frozen in liquid nitrogen; 10 μm sections were fixed in acetone and mounted. Images were acquired by fluorescence microscopy (Leica SP5) using Leica LAS-AF software (Leica Microsystems, Buffalo Grove, IL) and analyzed by ImageJ (NIH, Bethesda, MD). All animal studies have been approved by the Albert Einstein College of Medicine institutional review board.

**SUPPLEMENTARY MATERIAL**

**Figure S1.** Representative real-time PCR data from analysis of A9 uptake by LNCaP cells.

**Figure S2.** Cytometric analysis of clones identified from a doped-A9 internalization selection on HeLa-PSMA cells.

**Figure S3.** Cytometric analysis of clones identified from a doped-A9 internalization selection.

**Figure S4.** Sequences of functional clones identified from the A9 doped selection.

**Figure S5.** Sequence analysis of Round 6 of N62 internalization selection on HeLa cells.

**Figure S6.** Riboshredder is effective at destroying 2'-fluoro-modified RNA at the concentration and conditions used in the internalization selection.

**Figure S7.** Sequence analysis of Round 12 of the N50 internalization selection on Min66L1 cells.

**Figure S8.** Early fitness of the N62 pool internalization selection.

**Figure S9.** Retention of fluorescent signal of selected aptamers in cells after ribonuclease treatment.

**Figure S10.** Analysis of c1 binding to different mammalian cell lines.

**Figure S11.** HEK-EBNA 293 endocytosis inhibition.

**Figure S12.** Sequences tested for the minimization of c1.

**Figure S13.** Clones from separate selections compete for binding on cells and co-localize in A431 cells.

**Figure S14.** c1 is internalized into primary cells by clathrin-mediated transport.

**ACKNOWLEDGMENTS**

We would like to thank Simon Spivack for providing HBEC and NHBE cells and Jeffery Pessin for providing Min66L1 cells. This work was supported by grants from Stand Up 2 Cancer (SU2C-AACR-IRG0809), the Juvenile Diabetes Research Foundation (JDRF-S-2009-332), the Welch Foundation (F-1654), the National Institute of Health (TRO1 S 0R1A092839-01_02), the National Security Science and Engineering Faculty Fellowship (FA9550-10-1-0169), and the Susan G Komen Foundation (KGO81287 02). The authors declared no conflict of interest.

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