Development of cold switchable DNA aptamers for isolation of cells that express leukemia inhibitory factor receptor.

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Research report submitted to the Biomedical Sciences Program
in partial fulfillment of the requirements for the course
BIM 4009

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Ottawa, Ontario, Canada
April, 2015

ABSTRACT

Immunomagnetic cell sorting based on biomarker expression is an efficient procedure for isolation of large quantities of target cells from crude mixtures. However, this method is hindered by the limitations of antibody affinity ligands to elute target cells from the affinity matrix. Aptamers are developed to bind target receptor expressing cells by an *in vitro* cell SELEX procedure which can be modified to accommodate for viable cell elution from the affinity matrix in magnetic bead based cell sorting. Nucleic acids will form secondary structures with greater complexity of internal base pairing as solution temperature decreases. Therefore cooling has the potential to disrupt DNA aptamer affinity for a target receptor and allow elution of target cells during magnetic bead based affinity chromatography without the addition of exogenous agents. In this study a novel cold switchable cell SELEX method was developed to produce aptamers that bound to LIFR expressing HEK cells at 37°C and released these cells following incubation on ice. The downstream application of these cold switchable aptamers would be to use biotinylated forms of these affinity ligands to purify LIFR expressing target cells using streptavidin coated magnetic beads and elute the target cells by cooling the solution to 4°C. Three cold switchable aptamer candidates developed by the cold switchable cell SELEX procedure were screened by flow cytometry. The CS70 aptamer candidate exhibited significant binding to LIFR expressing HEK cells compared to the DNA library pool of randomly generated nucleic acid sequences. However, the three aptamer candidate sequences did not show selectivity for LIFR and therefore, appear to have affinity for another component on the HEK cell membrane. All aptamer candidates exhibited cold switchable activity, with the most promising aptamer candidate exhibiting 50% release from LIFR expressing HEK cells following incubation on ice. The novel cell SELEX procedure developed in this study has provided evidence for a new class of aptamers. With further optimization, the cold switchable cell SELEX protocol could provide an efficient method for developing affinity ligands that exhibit both selective binding and controlled release of target cells in aptamer based affinity chromatography cell isolation.

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to Dr. Maxim Berezovski for the opportunity to complete my honours research project in his Bioanalytical and Molecular Interaction (BioAMI) Laboratory at the University of Ottawa.

I would also like to thank Shahrokh Ghobadloo (PhD Candidate) for his mentorship and support as I completed my experiments.

I would like to thank all the members of the Dr. Berezovski group who welcomed me and supported my work.

Thank you especially to my parents Brian and Margaret McLaughlin for their unconditional love and support. Finally, I would like to thank my grandparents for their encouragement: Joan MacDonald and my dear late grandfather C.R. MacDonald, who I know is still cheering me on.

CONTRIBUTION STATEMENT

Conception:

Dr. Maxim Berezovski originated the idea of modifying the cell SELEX procedure to produce cold switchable aptamers for isolation of target cells in magnetic bead based affinity chromatography.

Experimental:

All experiments in this study were accomplished by Sarah McLaughlin with the following exceptions. LIFR expressing HEK recombinant cell line was developed by Shahrokh Ghobadloo (a Ph.D. candidate in the Berezovski laboratory). The twelfth round cell SELEX pool of selective LIFR binding DNA molecules, used as an enriched library for cold switchable cell SELEX performed by Sarah McLaughlin, was produced by Shahrokh Ghobadloo. Cold switchable aptamer candidate sequences were chosen in association with Shahrokh Ghobadloo from the LIFR cold switchable SELEX sixth round pool by Clustal alignment and phylogenetic tree analysis. LIF5 and LIF6 selective aptamers for LIFR expressing cells used as positive controls in this study were produced by Shahrokh Ghobadloo.

Writing:

This research report was written and edited by Sarah McLaughlin.

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1. INTRODUCTION

1.1 Aptamers: Chemically synthesized oligonucleotide affinity ligands

Oligonucleotides that bind ligands with high affinity were initially characterized *in vivo* with the discovery of riboswitches, which are components of an mRNA transcript that modulate protein expression through binding of intracellular substrates (1). Harnessing this knowledge, oligonucleotides termed aptamers were subsequently evolved *in vitro* to bind a target ligand determined by the experimenter (2). Aptamers are generated by the process of Systemic Evolution of Ligands by Exponential enrichment (SELEX) (3). The SELEX procedure is performed using a chemically synthesized DNA or RNA library of oligonucleotides that each contain a 40-100 base pair (bp) randomly generated region flanked by common 5' and 3' PCR primer binding sequences (3). For DNA aptamer development a DNA library typically contains 10^{14}-10^{15} sequences that each fold into a unique secondary structure (4). DNA aptamers are selected from this library through repetitive rounds of incubation with a target ligand, removal of non-binders and then PCR amplification only of DNA molecules that have affinity for the target of interest (3). Aptamers have been evolved to bind a wide variety of target ligands ranging from small organic molecules (dyes, antibiotics) and proteins (enzymes, gene regulators) to bacteria and viruses (5-10).

Aptamers exhibit sensitive and selective affinity for their target molecules similar to protein antibodies, which are the affinity ligands of choice for diagnostic procedures and cellular identification by biomarker analysis (4). Aptamers have several advantages as affinity ligands compared to their antibody counterparts. For example, SELEX procedure used to develop aptamers is performed *in vitro*, while antibody production occurs *in vivo*, therefore the optimal binding conditions between aptamer and target can be modified (4). Chemical modification of aptamers is also easier to accomplish, for example modified 2'-O-methyl pyrimidine nucleotides can be incorporated during SELEX improving stability by nuclease resistance (11). Aptamers unlike antibodies, can be evolved to bind molecules that are toxic or are not highly immunogenic and since nucleic acids are more stable than proteins they will not undergo

irreversible denaturation (4, 12). In addition, negative or counter selection can be performed to eliminate non-specific aptamer binding to a solid support used in affinity chromatography or a similar protein isoform of the target, thus taking advantage of synthetic evolution to improve target selectivity (13). Aptamers, which unlike antibodies are non-immunogenic, are also being developed as therapeutic agents (12). The first aptamer drug was approved for clinical use in 2004 to treat macular degeneration by blocking VEGF signaling (14). Furthermore, facile chemical modification of aptamers has made them candidates for cell-specific delivery of chemotherapeutic agents or siRNA molecules for prostate cancer therapy in xenograft models (15, 16).

1.2 The cell SELEX method

Aptamers like antibodies can be used to identify and isolate a cell sub-population based on biomarker expression. However, traditional SELEX methods were not always successful in developing aptamers for cell surface biomarkers because the isolated recombinant extracellular protein domains used as targets did not consistently retain their native configuration, contain post-translational modifications or mimic complex interactions of the membranome (17). The cell SELEX protocol was developed to select for aptamers that bind membrane protein biomarkers in their natural environment (18). Cell SELEX compares the binding of aptamers to a target cell line versus a control cell line in stages termed positive and negative selection, respectively (19). Only aptamers which bind the target cell line, and not the control cells, are amplified by PCR for use in further rounds of selection. Primer complimentary sites of library sequences are traditionally modified with a fluorescent label allowing for monitoring of cell SELEX DNA pool affinity for target cells by flow cytometry (19). Cell SELEX aptamer selection was originally performed using a target mammalian endothelial cell line, YPEN, with counter selection performed to eliminate aptamer affinity for glial cells (20). The resulting aptamer pool could bind selectively to blood vessels in rat glioblastoma brain tumors.

The cell SELEX procedure has been further adapted to select for aptamers which bind a specific biomarker overexpressed in the cytoplasmic membrane of a cell line that is amenable for transfection,

commonly the human embryonic kidney (HEK) cell line (21). In this adapted cell SELEX procedure only DNA molecules which bind to the recombinant HEK cell line overexpressing the target receptor and not the original HEK control cell line are amplified by PCR and subjected to further rounds of selection. Evolution of aptamer molecules which bind a specific cell surface biomarker in its native conformation have been used in cell isolation by fluorescence activated cell sorting (FACS) technology (22). For example, cell SELEX generated aptamers for murine c-kit receptor enabled better FACS yield of murine c-kit positive hematopoietic progenitor cells compared to the murine c-kit antibody (21).

1.3 Aptamer affinity chromatography for isolation of cells based on cell surface biomarker expression

FACS cell separation is a sensitive method yielding cell sub-populations of high purity and can sort cells into multiple classes based on the expression of numerous biomarkers (23). However, it is also an expensive technology and difficult to scale up to purify large quantities of target cells from crude samples (24). For this reason affinity chromatography methods, such as immunomagnetic cell sorting, have become the cell isolation methods of choice for many laboratories (25). Immunomagnetic cell isolation is a simpler cell sorting procedure using antibody coated magnetic beads for isolation of large quantities of cells expressing a target receptor of interest and is at times used as a preparatory step for the more sensitive FACS analysis (25). The first major application of immunomagnetic sorting was the detection of breast tumor cells in bone marrow for cancer diagnosis (26). Aptamers have advantages over antibodies in affinity chromatography-based cell isolation applications due to their greater stability, smaller size that allows high ligand density on an affinity matrix and flexible modification for attachment to a solid support, for example biotin labeled aptamers that can bind with high affinity to streptavidin coated beads (27). An area of difficulty in current antibody and aptamer based magnetic bead isolation of cells is the release of the target cells expressing a receptor of interest from the affinity matrix (27).

Removal of antibody or aptamer functionalized beads from cells ensures cells are not modified by the isolation process, as bound affinity ligands can cause activation of intracellular signaling pathways or

iron oxide cytotoxicity (25, 28). One of the most commonly used methods of antibody-magnetic bead removal from target cells is the development of antibodies which target the FAB fragment of the antibody that binds the target receptor, thereby eluting target cells through competitive binding (29). However, this technology requires the development of an elution antibody and an affinity antibody for each biomarker based cell isolation procedure and the recovery yield of target cells can be hindered by high affinity between the biomarker and its antibody (30). Elution strategies for aptamer coated magnetic bead cell isolation have included exonuclease aptamer digestion, however contamination of isolated cells with exonuclease can hinder genomic analysis of isolated cells without stringent purification (31). Therefore, more effective elution strategies releasing target cells from aptamers that do not place limitations on analyses of the isolated cell population are desirable to develop a superior magnetic bead cell isolation assay compared to the current antibody based platform.

Temperature, salt concentration and pH are three key determinants of aptamer secondary structure and therefore target affinity, which can be modified to facilitate viable cell elution. Aptamers have the unique advantage of being synthetically evolved to bind a target, therefore the conditions of cell SELEX can be modified to accommodate release strategies at different buffer conditions, pH or temperature (32). Structure switching aptamers have been selected with traditional SELEX, such that these aptamers elute bound human L-selectin protein using the chelating agent EDTA with an 83% yield of L-selectin target (33). Current cell SELEX methodology uses heating to 95°C to release bound aptamers from their cell surface receptor targets (19), which is not a release method that can be used to isolate viable cells. Switchable aptamers, proposed by our research group, can be developed by changing release conditions in the cell SELEX protocol, only amplifying aptamers for further selection if they release the target cell surface biomarker under conditions desirable for the elution phase of cell isolation procedures (Figure 1). For example, the concentration of calcium and magnesium ions in a binding buffer help stabilize the secondary structure of nucleic acid aptamers with observed aptamer conformational changes due to salt concentration occurring *in vivo* (34). Switchable aptamers have been evolved to

release their targets by the addition of EDTA and EGTA chelating agents allowing purification of oncolytic viruses by affinity chromatography (Figure 2) (30).

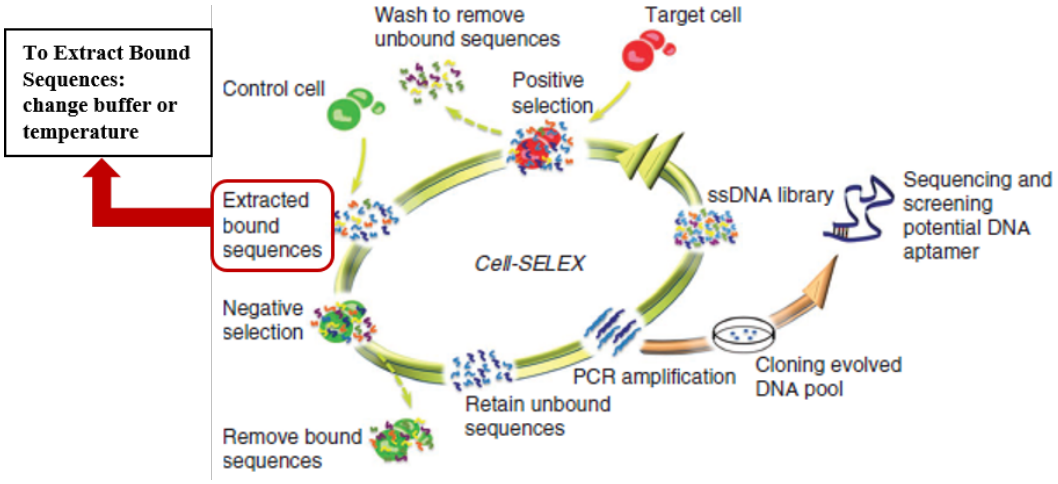


Figure 1. The cell SELEX protocol can be adapted for efficient elution of target cells for aptamer affinity chromatography cell based isolation (Modified from (19)). Changing the conditions of target cell receptor release during the cell SELEX protocol to alternate buffer conditions or lower temperature incubation allows development of aptamers which elute target cells under conditions desirable for aptamer affinity chromatography based cell isolation.

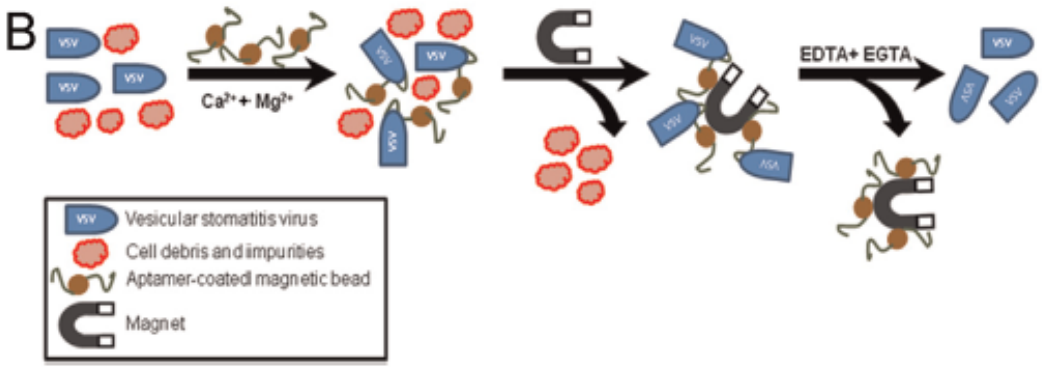


Figure 2. Schematic representation for magnetic bead based isolation of oncolytic viruses with switchable aptamers previously established in our laboratory (30). Aptamers were selected using a modified cell SELEX procedure to bind vesicular stomatitis virus at room temperature in PBS buffer with Ca^{2+}/Mg^{2+} and then release these viral targets with the addition of EDTA and EGTA chelating agents that destabilize aptamer secondary structure. Therefore, using streptavidin coated magnetic beads and

biotinylated aptamer, harvested VSV viruses were successfully purified from cell debris and eluted from the aptamer-magnetic bead complex through the addition of EDTA/EGTA.

1.4 Temperature dependent elution strategies for aptamer based cell isolation

Temperature dependent elution strategies for aptamer affinity chromatography offer an intrinsic release method that does not require the addition of external chemicals or biomolecules that could contaminate or modify isolated target cells prior to downstream analyses. Aptamers have been selected to bind their targets only at a specific temperature by adjusting the conditions of SELEX (4). The earliest application of thermal switchable aptamers relied on reversible denaturation of aptamer secondary structure at high temperature allowing target release (35). Aptamers specifically designed for thermal release of ATP target showed regeneration of the affinity matrix at 30-40°C, which was more efficient than a competitive elution strategy commonly used in antibody affinity chromatography (36). In order to isolate viable cells through affinity chromatography, temperature dependent elution from aptamers must not affect cell viability. Therefore raising solution temperature for elution above physiological temperature of 37°C is not an applicable strategy.

One alternative strategy to accomplish temperature dependent aptamer release is cooling dependent elution. Aptamers would be selected in cell SELEX to bind target receptor expressing cells at 37°C then release these cells by lowering the incubation temperature to 4°C. Cells retain their viability for moderate incubation periods at 4°C as cellular enzymatic reactions do not proceed at their normal rate, thereby inducing a state of metabolic stasis. Studies of nucleic acid secondary structure have determined that lowering the temperature of solution allows the increased formation of complex motifs (including loops, bulges and junctions) that are not stable at higher temperatures, including less stable tertiary interactions within the oligonucleotide itself (37). Therefore, structural rearrangements within the aptamer secondary structure could occur during incubation at 4°C that would disrupt binding between aptamer and target membrane receptor, allowing cell isolation and release. While low temperature aptamer release has

not been accomplished before, thermal sensitive aptamers have been developed to bind targets specifically at physiological temperature and show limited binding to their target at 10°C above or below this temperature (38). It is anticipated that this novel approach for affinity chromatography-based cell isolation will have limited impact on cell viability, cellular expression profile and will not interfere with downstream functional analysis of the isolated cells.

1.5 Cold temperature dependent switchable aptamers for LIFR expressing cell isolation

The cell SELEX target biomarker used in this study for development of cold switchable aptamers was leukemia inhibitory factor receptor (LIFR). LIFR is a cytoplasmic membrane receptor that heterodimerizes with gp130 general interleukin 6 receptor to bind leukemia inhibitory factor (LIF) cytokine (39). A soluble form of LIFR also serves as an LIF signaling antagonist in the blood stream (40). LIFR was named due to the original function of LIF signaling through this receptor to induce differentiation of myeloid leukemia cells, and thereby reducing leukemic tumor expansion (41). The LIFR transmembrane receptor will initially bind with low affinity (nM range) to the LIF ligand through a central Igg like extracellular domain (42). The gp130 cytokine receptor then binds the LIF ligand in the LIF:LIFR complex with picomolar affinity to form a trimeric signaling complex (43). Dimerization of gp130:LIFR receptors by LIF binding allows janus kinase (JAK) phosphorylation of the intracellular domain of the gp130 subunit which phosphorylates and activates signal transducer and activation of transcription 3 (STAT3) mediating expression of STAT3 target genes (44) (Figure 3).

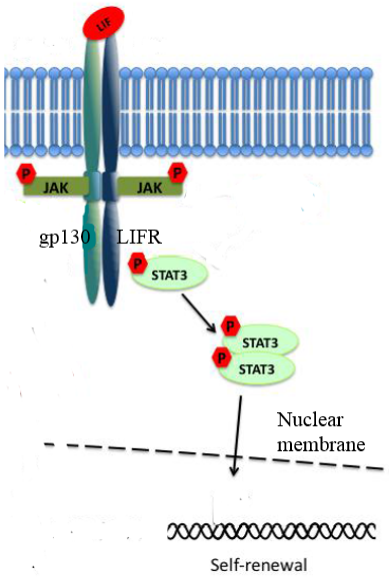


Figure 3. LIF signaling through the LIFR:gp130 heterodimer activating STAT3 (Modified from (45)). STAT3 transcription regulator translocates to the nucleus and induces target gene expression primarily involved in the upregulation of stem cell self-renewal pathways.

The major signaling pathway transduced by LIFR is thus mediated by LIF, a heavily glycosylated single chain polypeptide cytokine of the interleukin six family (46, 47). A major role of the LIF:LIFR signaling axis is the maintenance of embryonic stem cell pluripotency by inhibiting differentiation along endoderm and mesoderm linages through STAT3 transcription factor signaling (48, 49). Expression of LIF by the extraembryonic section of the egg cylinder and LIF:LIFR signaling is implicated in early embryogenesis, including implantation and maintenance of a microenvironment promoting self-renewal in the developing blastocyst (50, 47). Studies have also shown that enhanced LIF mediated STAT3 signaling increases LIFR expression that leads to an increase in tumor cell growth and metastasis in pancreatic carcinoma, melanoma and nasopharyngeal carcinoma (51-53). The established role of LIF signaling in embryonic self-renewal and function in cancer progression corroborate the finding that tumors with embryonic-like signatures are more aggressive with greater recurrence rates (54).

Due to its upregulation in several metastatic cancers, LIFR has been postulated to play a role in self-renewal of a sub-population of tumor cells with stem cell properties that have been termed cancer stem cells (CSCs) (55). CSCs are functionally defined as a distinct subpopulation of tumor cells which have the ability to regenerate the original tumor in a xenograft model (56). CSCs have shown chemotherapy resistance leading to tumor recurrence and have been associated with cancer metastasis (57, 58). In glioblastoma CSCs, TGF-β growth factor signaling induces LIF expression which through JAK-STAT3 signaling controls CSC self-renewal and oncogenic capacity in vivo (59). However, LIFR is down regulated in metastatic breast cancer tissue and was characterized as a breast cancer metastasis suppressor which is contradictory to its hypothesized role in CSC self-renewal (60).

The cold switchable aptamers developed in this study could facilitate the elucidation of these contrasting results. In order to determine the role of LIFR expression in CSC self-renewal, viable LIFR expressing tumor cells need to be isolated. A key component of the CSC hypothesis is that a tumor can be fractionated based on self-renewal capacity (61). Previously, the cell SELEX approach was used to develop aptamers that targeted prostate tumor cells with enhanced self-renewal capacity and aptamers could which enrich for brain cancer stem cells (62, 63). Low temperature dependent switchable aptamers targeting LIFR could allow efficient magnetic bead based isolation of LIFR expressing tumor cells through 4°C elution of target cells. Functional assays using these isolated cells can be used to determine whether they are enriched for tumorigenic activity and self-renewal capacity, through xenograft or *in vitro* sphere forming assays, respectively (57). Therefore, cold switchable aptamers developed in this study have the potential to facilitate isolation of viable LIFR expressing tumor cells, unaltered by the separation method, which can aide in resolving the contradictory roles of LIFR in cancer stem cell self-renewal (Figure 4).

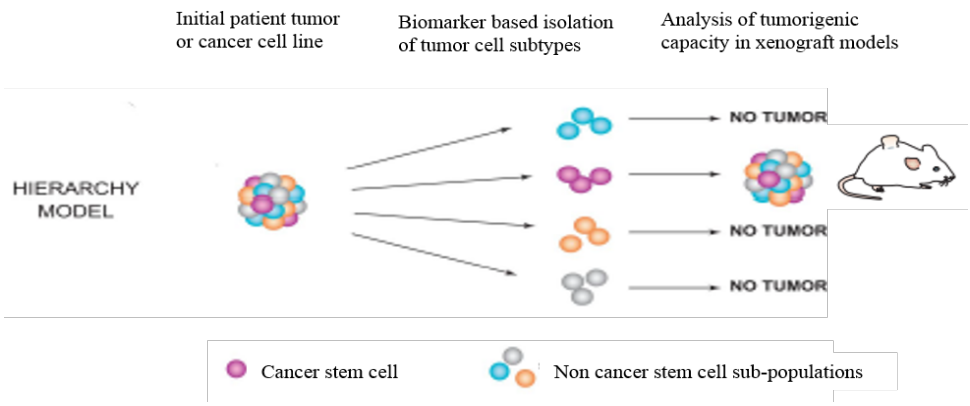


Figure 4. Cancer stem cell biomarkers allow isolation of tumor cell fractions which are enriched for *in vivo* tumor initiating capacity according to the hierarchal model of tumor heterogeneity (Modified from 56, 58). In order to determine if LIFR plays a role in CSC self-renewal, cold switchable aptamers can be developed which isolate viable LIFR expressing tumor cells. These aptamers would allow the study of isolated LIFR expressing cells in mouse xenograft assays to identify if they are enriched for tumorigenic capacity, an indicator of CSC self-renewal, compared to LIFR negative cancer cells.

Due to the propensity of oligonucleotides to form more complex motifs with internal base pairing as the temperature of a solution decreases, it is predicted that the cell SELEX procedure can be modified to produce aptamers that change conformation and release target receptor expressing cells following incubation on ice. These cold switchable aptamers would therefore provide an improved elution strategy for cell isolation by aptamer based affinity chromatography. The objective of this study was to use a modified cell SELEX procedure, termed cold switchable SELEX, to evolve DNA aptamers that selectively bind LIFR overexpressing HEK cells at 37°C and release these target cells following incubation on ice. The secondary objective was to use biotinylated forms of these aptamers in a streptavidin coated magnetic bead assay to isolate LIFR overexpressing HEK cells from a heterogeneous mixture. If successful, the developed LIFR specific aptamer sequences have potential use in further isolation of LIFR expressing cells in tumor cell lines or patient samples. Due to the lack of exogenous contaminants or physical stress imposed by the cold switchable elution strategy, isolated cell fractions could be used directly in functional assays to evaluate the potential of LIFR as a biomarker for CSC enrichment and to study its functional role in CSC self-renewal.

2. METHODS

2.1 Cell lines and culture

Overexpression of Leukemia Inhibitory Factor Receptor (LIFR) in Human Embryonic Kidney (HEK) 293T cell line (Clontech) was established in our laboratory using the Lenti-X Tet-On Inducible Expression System (Clontech). Briefly, HEK 293T cells were transduced with a pLVX-TRE3G-LIFR response vector and a pLVX-Tet3G regulator vector. The response vector contains the human LIFR gene under the control of the TET-On 3G response element promoter (pTRE3G), while the regulator vector constitutively expresses TET-On 3G protein from the human PGK promoter. In the presence of the co-activator doxycycline (Clontech), TET-On regulator protein binds to its response element pTRE3G and activates transcription of the downstream LIFR gene to establish the LIFR overexpressing HEK cell line (LIFR-HEK).

LIFR-HEK and HEK cell lines were maintained in media containing 90% high glucose Dulbecco's Modified Eagle's Medium (DMEM) with 4.0 mM L-glutamine and 1 mM sodium pyruvate (Hyclone, Thermo Scientific), and supplemented with 10% fetal bovine serum (FBS) (Hyclone, Thermo Scientific). For the LIFR-HEK cell line, media was also supplemented with 1 µg/mL doxycycline to maintain LIFR expression. Cells were passaged every 3-4 days or once they reached 90% confluency as determined by light microscopy (Micromaster, Fisher Scientific). To passage cells, they were washed with Phosphate Buffered Saline (PBS) buffer without Ca^{2+} or Mg^{2+} (Hyclone, Thermo Scientific), removed from the culture dish using 0.25% trypsin – 0.1% EDTA solution (Hyclone, Thermo Scientific) and finally trypsin activity was neutralized by the addition of 10% FBS in DMEM media. All cell lines were grown in a Heracell 150i CO_2 incubator (Thermo Scientific) environment with 5% CO_2 and a temperature of 37°C.

2.2 Preparation of LIFR-HEK and HEK cell samples for flow cytometry assays

LIFR-HEK and HEK cell plates were harvested for flow cytometry assays when they had grown to 80-90% confluency as assessed by light microscopy. To dissociate cells from the culture plate they were first incubated for 5 min in 10 mL of PBS without Ca^{2+}/Mg^{2+} (Hyclone, Thermo Scientific). The PBS buffer was removed by aspiration and then the cells were incubated in 0.5 mL of 0.25% trypsin – 0.1% EDTA solution for 30 sec. Finally, DMEM media supplemented with 10% FBS was added to a volume of 10 mL. The cells were disaggregated using a mechanical pipette and the resulting single cell suspensions were transferred to a Falcon tube. Cell suspensions were pelleted by centrifugation at 200 x g and 4°C for 3 min in a table top centrifuge (Sigma 3-16K, Montreal Biotech). LIFR-HEK and HEK cell pellets were washed by resuspending the cell pellets in 2 mL of PBS with Ca^{2+}/Mg^{2+} followed by centrifugation at 200 x g and 4°C for 3 min. Following the wash procedure, cell pellets were resuspended in 2 mL of PBS with Ca^{2+}/Mg^{2+}. LIFR-HEK and HEK cells were counted using the Count and Viability Assay for the MUSE Cell Analyzer (EMD Millipore). Based on the viable cell count the LIFR-HEK and HEK cell suspensions were adjusted to a final concentration of $2x10^6$ cells/mL in PBS buffer with Ca^{2+}/Mg^{2+}.

2.3 Validation of LIFR expression in transduced LIFR-HEK cell line by flow cytometry

Mouse anti-human LIFR monoclonal antibody conjugated to Alexa Fluorophore 700 (AF700; BD Pharmigen) was added at a concentration of 5 µg/mL to both a LIFR-HEK cell sample and a HEK cell sample, with each sample containing 200,000 cells prepared in PBS with Ca^{2+}/Mg^{2+}. The anti-LIFR antibody was allowed to incubate with the cell samples at 4°C for 60 min in the dark. The cell samples were then washed in PBS buffer with Ca^{2+}/Mg^{2+}. All washing procedures for flow cytometry cell samples were performed as follows: cell suspensions were pelleted by centrifugation at 200 x g and 4°C for 3 min in a Lab Net Prism R microcentrifuge (Montreal Biotech), the supernatant was aspirated, cell pellets were re-suspended in 400 µL of PBS with Ca^{2+}/Mg^{2+} and cells were pelleted once again by centrifugation at 200 x g and 4°C for 3 min. The LIFR-HEK and HEK anti-LIFR stained cell samples were resuspended in 400 µL of PBS with Ca^{2+}/Mg^{2+} for flow cytometry analysis. Two additional non-stained cell samples

containing 200,000 cells of LIFR-HEK or HEK cell lines were prepared in 400 µL of PBS with Ca^{2+}/Mg^{2+} as non-stained control samples for flow cytometry.

The AF700 fluorescence intensity of each cell sample was analyzed using a Gallios Flow Cytometer (Beckman Coulter). For each sample 10,000 events were collected within a gate defined by the approximate size and granularity of the live HEK cell population based on a side scatter logarithmic versus forward scatter linear dot plot in the acquisition protocol (Gate A, Figure 6B). Expression of LIFR in the LIFR-HEK cell line was analyzed using Kaluza software (Beckman Coulter) to determine the median AF700 fluorescence intensity of each cell sample, which correlates to anti-LIFR antibody binding to LIFR. The fold difference in MFI-AF700 emitted from the LIFR-HEK cell line compared to the untransduced HEK cell line was calculated, after both AF700 MFI values had been corrected for cell autofluorescence using the non-stained cell samples.

2.4 PCR amplification of a cell SELEX pool enriched with LIFR-binding aptamers

In our laboratory, twelve rounds of cell SELEX were performed as described by Selah et.al. (19) to develop an aptamer pool that selectively bound LIFR cell surface biomarker. The LIFR-HEK cell line was used as the target cells for positive selection of aptamers at 37°C and the HEK cell line served as the control cell line to remove aptamers (negative selection) that were non-selective binders for LIFR at room temperature. Three cell SELEX rounds of positive selection followed by nine cell SELEX rounds containing both positive and negative selection were completed to develop the pool of DNA molecules enriched with LIFR binding aptamers. This pool was evolved from the Havard single stranded DNA library (Integrated DNA Technologies). This library contained 10^{15} unique single stranded DNA molecules each of which were 100 base pairs in length and contained two PCR primer consensus sequences at the 5' and 3' ends of a randomly generated 60 base pair region that was unique to each individual molecule. All the DNA molecules in the library were labelled with a Cy5 fluorophore at the 5' end.

The twelfth round SELEX pool of selective LIFR binding DNA molecules was used as an enriched library for cold switchable (CSW) aptamer selection. In preparation for the first round of cold switchable cell SELEX, PCR amplification of DNA molecules recovered from the twelfth round of cell SELEX for LIFR was performed with Cy5 labeled forward primer: 5'-CY5- CTC CTC TGA CTG TAA CCA CG-3'(Integrated DNA Technologies) and a 5' phosphorylated reverse primer: 5'- P-GGC TTC TGG ACT ACC TAT GC-3'. The PCR reaction mixture contained: 1X PHIRE reaction buffer with 1.5 mM $MgCl_2$ (Thermo Scientific), 200 µM of dNTPs (Thermo Scientific), 0.5 µM of Cy5-forward primer, 0.5 µM of phosphorylated reverse primer, 0.02U/µL of PHIRE Hot Start II DNA polymerase (Thermo Scientific), 10% DMSO (Thermo Scientific) and 35 ng of purified template DNA recovered from cell SELEX. PCR reaction conditions were: initial denaturation for 5 min at 98°C, 35 cycles of denaturation at 98°C for 15 sec, annealing at 56°C for 20 sec and extension at 72°C for 10 sec, which was followed by a final extension stage at 72°C for 1 min. The 5' phosphorylated reverse complimentary strands of the PCR amplified SELEX pool DNA product were digested by Lambda exonuclease enzyme (Thermo Scientific) to recover the single stranded aptamer candidate molecules from the original SELEX pool. For each exonuclease digestion reaction 4U of exonuclease was added to 200 µL of PCR product in 1X Lambda buffer (670 mM glycine-KOH (pH 9.4), 25 mM $MgCl_2$, 0.1% (v/v) Triton X-100) (Thermo Scientific). Exonuclease digestion of the SELEX PCR product required incubation in a thermocycler at 37°C for 4 h.

2.5 Cold Switchable cell SELEX (CSW SELEX)

The first round of positive selection using CSW SELEX was performed with 200 nM of exonuclease digested PCR product from the LIFR twelfth round SELEX pool. The objective of CSW SELEX was to select from this pool DNA molecules that bind LIFR at 37°C and release from LIFR expressing target cells at 4°C, as a result of changing conformation (Figure 5). The single stranded DNA pool was added to 1 mL of PBS with Ca^{2+}/Mg^{2+} and denatured at 95°C for 10 min followed by snap cooling at -20°C for 5 min. This procedure helps to recover the unique secondary structure of each potential aptamer sequence in the pool prior to selection. Media was aspirated from a 100 mm x 20 mm

cell plate (BioLite, Thermo Scientific) containing 90-95% confluent LIFR-HEK cells (approximately $9x10^6$ cells) and the DNA solution prepared from the LIFR twelfth round SELEX pool was added dropwise to the cells using a 1000 µL pipette. For positive selection, the cells were incubated with the SELEX pool DNA on a slow rocker (200 rpm) at 37°C for 60 min. Following incubation the cells were removed from the cell culture plate in 4 mL of PBS with Ca^{2+}/Mg^{2+} using a 1000 µL pipette. The cell suspension was centrifuged at 200 x g and 20°C for 3 min. The supernatant containing weak binding DNA molecules was aspirated and the target LIFR-HEK cell pellet with bound DNA was washed by resuspending the pellet in 4 mL of PBS with Ca^{2+}/Mg^{2+} followed by centrifugation at 200 x g and 20°C for 3 min. The cell pellet was resuspended in 50 µL of PBS with Ca^{2+}/Mg^{2+} and incubated on ice for 30 min. Following incubation the cell suspension was pelleted by centrifugation at 200 x g and 4°C for 3 min and the supernatant containing DNA molecules that released from the LIFR-HEK cells during incubation on ice was recovered.

For negative selection using CSW cell SELEX, a HEK cell pellet previously prepared from a 95% confluent 100 mm x 20 mm culture plate (approximately $9x10^6$ cells) was resuspended with 50 µL of the supernatant containing DNA molecules released from the LIFR-HEK cell line at 4°C. This cell suspension was incubated at room temperature for 30 min and then centrifuged at 1000 x g and 4°C for 10 min to remove DNA molecules which bound to components of the HEK cell membrane and therefore were not selective for LIFR. The final supernatant recovered from CSW SELEX was expected to contain DNA molecules that bind to LIFR-HEK cells at 37°C and released from these LIFR expressing cells at 4°C.

Potential aptamer candidates were isolated from the CSW SELEX supernatant by agarose gel electrophoresis. CSW SELEX supernatant was mixed with 1X DNA Gel Loading Dye (Thermo Scientific) and run on a 1% agarose gel (1X TAE buffer, Ultrapure agarose (Life Technologies) and 2 µL of GelRed (Biotium)), along with MassRuler Low Range Molecular Weight DNA ladder (Thermo Scientific, SM0383) and a previously PCR amplified SELEX aptamer pool as a positive control. The

agarose gel was run at 160V for 20 min. The DNA band from the CSW SELEX supernatant running an equal distance as the 100 bp ladder band (Havard library DNA molecule length) and the positive control aptamer band was visualized by UV light in a FluorChem Q imaging system (Alpha Innotech) and excised from the gel using a surgical blade. The CSW SELEX pool DNA was purified from the gel slice using a GeneJet Gel Extraction Kit column (Thermo Scientific) according to the manufacturer's protocol. The CSW SELEX pool was eluted from the column by incubating 30 µL double distilled water (Synergy UV Millipore) directly on the filter at 50°C for 30 min and centrifuging the column at 12,000 x g and 40°C for 5 min. The concentration of the single stranded DNA in the recovered CSW SELEX pool was measured using a NanoDrop 200 UV-Vis Spectrophotometer (Thermo Scientific). PCR amplification of the CSW SELEX pool DNA as described in section 2.6 was used to produce an enriched DNA pool for the next round of CSW SELEX. Each round of CSW SELEX was intended to increase the stringency of selection for aptamers that bound LIFR at 37°C and released from LIFR expressing cells at 4°C. Six rounds of CSW SELEX were performed including four rounds with positive selection and cold release, followed by two rounds with positive selection, cold release and negative selection.

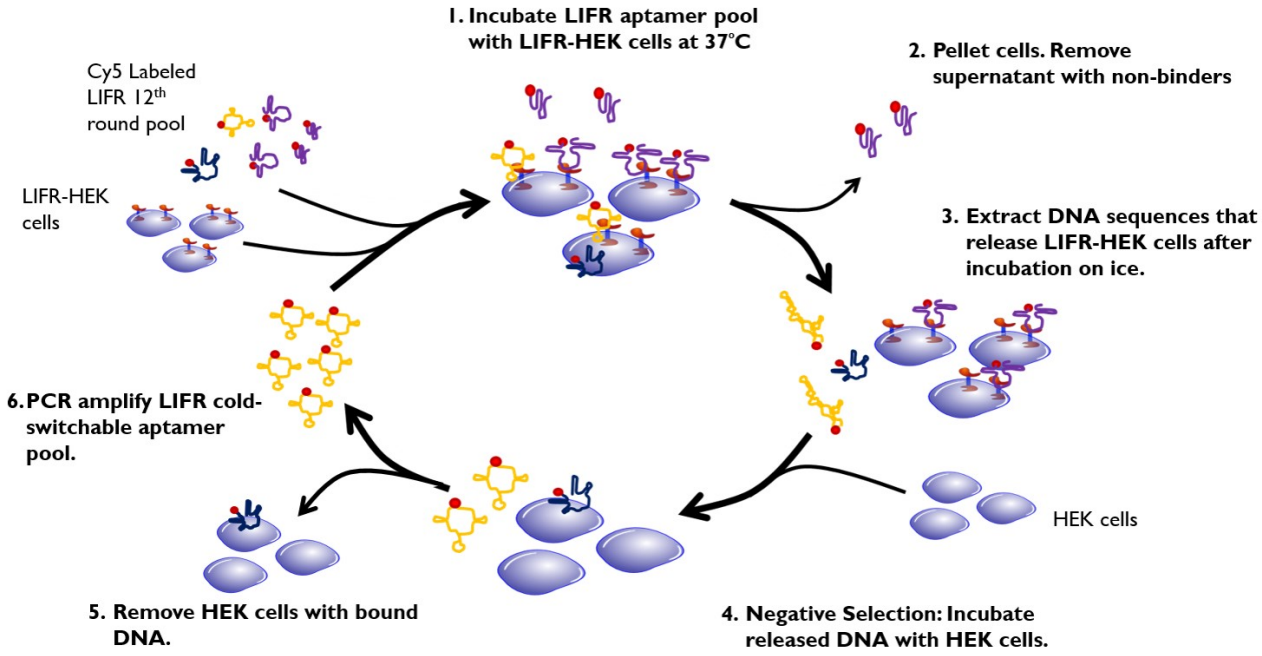


Figure 5. Cold-switchable SELEX procedure targeting leukemia inhibitory factor receptor (LIFR) expressing cells. A modified cell SELEX procedure was used to develop LIFR selective aptamers which bind to target expressing cells at 37°C and then release these cells following incubation on ice. The original pool of DNA molecules used for selection was a twelfth round cell SELEX pool of 100 bp DNA ligands evolved to selectively bind LIFR expressing cells. LIFR overexpressing recombinant Human Embryonic Kidney (LIFR-HEK) cell line was used as the target cell line for positive selection. HEK untransduced control cell line was used for negative selection to remove non-selective binders. PCR amplified DNA molecules recovered from each CSW SELEX round were used for the following round of selection. A total of six CSW SELEX rounds were performed, including four rounds with positive selection and ice release, followed by two rounds with positive selection, ice release and negative selection stages.

2.6 PCR amplification of CSW SELEX pools

PCR amplification of DNA molecules recovered from all CSW SELEX pools was performed with Cy5 labeled forward primer: 5'-CY5- CTC CTC TGA CTG TAA CCA CG-3' and 5' phosphorylated reverse primer: 5'- P-GGC TTC TGG ACT ACC TAT GC-3'. Due to low recovery yield of DNA from the first round of CSW SELEX two PCR reactions were performed. The initial PCR reaction mixture contained: 1X PHIRE reaction buffer with 1.5 mM $MgCl_2$, 200 µM of dNTPs, 0.5 µM of Cy5-forward primer, 0.5 µM of phosphorylated reverse primer, 0.02U/µL of PHIRE Hot Start II DNA polymerase, 5% DMSO and 31 ng of purified template DNA recovered from CSW SELEX. Thermocycler program conditions for PCR were: initial denaturation for 5 min at 98°C, 30 cycles of denaturation at 98°C for 15 sec, annealing at 58°C for 20 sec and extension at 72°C for 10 sec, followed by a final extension at 72°C for 1 min. The second PCR reaction mixture contained the same reagents as above except only 1% DMSO was added and 2 µL of the first PCR product served as the template. Thermocycler program conditions for PCR were the same as the original PCR reaction except 56°C was use as the annealing temperature to adjust for lower DMSO concentration.

The second and third CSW SELEX pools were amplified using an optimized reaction mixture of 1X PHIRE reaction buffer with 1.5 mM $MgCl_2$, 200 µM of dNTPs, 0.5 µM of Cy5-forward primer, 0.5 µM of phosphorylated reverse primer, 0.02U/µL of PHIRE Hot Start II DNA polymerase, 3% DMSO and 80 ng of purified template DNA recovered from cell SELEX. The thermocycler program was the same as

that used for the first round CSW SELEX PCR reaction, except with a 56°C annealing temperature. Finally, for the fourth through sixth round CSW SELEX pools the optimized PCR reaction mixture used to reduce non-specific amplification contained: 1X Bestaq Master Mix (ABM), 0.5 µM of barcode-forward primer, 0.5 µM of phosphorylated reverse primer and 80 ng of CSW SELEX pool DNA template. The thermocycler program for the PCR reactions was: initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 10 sec, annealing at 55°C for 30 sec and extension at 72°C for 12 sec, which was followed by a final extension at 72°C for 1 min. For all PCR reactions a negative control was performed with the same reaction conditions except an equal volume of double distilled water was added instead of CSW SELEX pool DNA template.

All PCR reaction products were confirmed by agarose gel electrophoresis using a 3% agarose gel made with TAE buffer and run at 160 V for 30 min. Samples resolved by electrophoresis included: PCR amplified CSW SELEX pool DNA, negative PCR control and a positive control of previously amplified cell SELEX pool DNA, with all samples in 1X DNA loading dye. The target DNA band containing potential aptamer candidates from the CSW SELEX pool DNA was 100 bp (Havard library DNA molecule length) and was visualized in the Cy5 filter of the FluorChem Q imaging system. Gel pictures were taken and analyzed with Alphaview software (Protein Simple).

To prepare the CSW SELEX PCR product for another round of selection, the 5' phosphorylated reverse complimentary strands were digested by Lambda exonuclease enzyme to recover the single stranded potential aptamer candidate molecules from the original CSW SELEX pool. Exonuclease digestion was carried out using the same protocol as described in section 2.5 for the PCR amplified twelfth round LIFR cell SELEX pool.

2.7 Flow cytometry analysis of DNA molecules selected by CSW SELEX

To screen the DNA molecules selected after six rounds of CSW SELEX a flow cytometry assay was performed to determine the ability of the Cy5 labeled DNA molecules to bind selectively to LIFR

expressing cells at 37°C and release from these cells at 4°C. In preparation for flow cytometry, the CSW SELEX sixth round (CSW6) pool was PCR amplified with Cy5 containing forward primer and exonuclease digested as described in section 2.6. A cell sample containing a LIFR-HEK cell concentration of $1x10^6$ cells/mL and 200 nM of the single stranded DNA from the CSW6 pool in a 400 µL volume of PBS with Ca^{2+}/Mg^{2+} was incubated at 37°C for 60 min. The cell suspension was then centrifuged at 200 x g and 20°C for 3 min, washed and resuspended in 400 µL of PBS with Ca^{2+}/Mg^{2+}. This cell suspension containing LIFR-HEK cells with bound CSW6 pool DNA was then divided into two 200-µL aliquots (200,000 cells each). One 200 µL aliquot of the LIFR-HEK cell sample was made up to 400 µL in PBS with Ca^{2+}/Mg^{2+} in preparation for flow cytometry analysis. The second 200-µL aliquot of LIFR-HEK cell sample was incubated on ice for 30 min to analyze the cooling dependent release of CSW6 pool DNA molecules. This cell sample termed CSW6 ice release, was pelleted at 200 x g and 4°C for 3 min and the supernatant containing any released DNA molecules was discarded. The CSW6 ice release sample cell pellet was resuspended in 400 µL of PBS with Ca^{2+}/Mg^{2+} in preparation for flow cytometry analysis.

Several control samples were also prepared for flow cytometry analysis. To assess selectivity of the sixth round pool DNA for binding to LIFR, 200 nM of the DNA molecules were incubated with $1x10^6$ cells/mL HEK cells, which do not have the LIFR receptor, in a 200 µL volume of PBS with Ca^{2+}/Mg^{2+} at 37°C for 60 min. As a negative binding control 200 nM of Havard Cy5 labeled DNA library was incubated with $1x10^6$ cells/mL LIFR-HEK cells in a 200 µL volume of PBS with Ca^{2+}/Mg^{2+} at 37°C for 60 min. For these controls, cells were washed and resuspended in 400 µL of PBS with Ca^{2+}/Mg^{2+}. Finally, non-stained controls for cell autofluorescence were prepared with 200,000 LIFR-HEK or HEK cells alone in 400 µL of PBS with Ca^{2+}/Mg^{2+}.

The Cy5 fluorescence of all cell samples were analyzed using a FC500 flow cytometer (Beckman Coulter) in the FL4 detector. Flow cytometry data analysis was performed using Free Flowing software (Perttu Terho, Turku Center for Biotechnology). The live cell gate was set on the main population in the

LIFR-HEK non-stained control side scatter logarithmic versus forward scatter linear dot plot. The Cy5 median fluorescence intensity of each experimental sample is based on the gated live cell population and was corrected for autofluorescence using the Cy5 median fluorescence intensity of the cells alone control.

2.8 Sequencing of DNA molecules from the sixth round CSW SELEX pool and selection of DNA aptamer candidates

Following flow cytometry screening of selective LIFR expressing cells binding at 37°C and release of these cells after incubation on ice, the CSW6 DNA pool was prepared for sequencing. The original DNA purified from the sixth round of CSW SELEX was amplified by PCR using barcode containing forward primer: 5'-TGACTGAC-CTC CTC TGA CTG TAA CCA CG-3' and the 5' phosphorylated reverse primer: 5'- P-GGC TTC TGG ACT ACC TAT GC-3'. The PCR reaction conditions were the same as those used to amplify the CSW6 pool for flow cytometry analysis (section 2.6). The sixth round CSW SELEX pool PCR product and negative PCR control were mixed with 1X DNA loading dye and run on a 1% agarose gel (1X TAE buffer, Ultrapure agarose and 2 µL of GelRed) along with MassRuler low range molecular weight DNA ladder and previously amplified SELEX aptamer pool as a positive control. Electrophoresis was run for 45 min at 160 V and the potential aptamer candidates for sequencing were excised from the gel using UV lamp visualization in the FluorChem Q imaging system. The CSW SELEX pool DNA was purified from the gel slice using a GeneJet Gel Extraction Kit column according to the manufacturer's protocol. The sixth round CSW SELEX pool was eluted from the column by incubating 30 µL double distilled water (Synergy UV Millipore) directly on the filter for 30 min at 50°C and centrifuging the column for 5 min at 12,000 x g and 40°C. The concentration of the DNA in the recovered sixth round CSW SELEX pool was measured using a NanoDrop 200 UV-Vis Spectrophotometer to ensure sufficient product for DNA sequencing.

An 80 ng sample of sixth round CSW SELEX for LIFR PCR product was sequenced by Eurofins Genomics using Illumina MiSeq next generation sequencing. Sequencing analysis for the sixth round CSW SELEX pool was performed using Galaxy software (64, 65) FASTAQ to FASTA then FASTA

collapse tools to list unique DNA sequences by abundance. To isolate DNA sequences from the sixth round CSW SELEX pool, as multiple aptamer pools were sequenced in the same analysis, the collapsed FASTA data was converted to Tabular format to allow for selection of sequences only containing the CSW SELEX specific 8 bp barcode and common forward primer sequence (5'-TGACTGAC-CTC CTC TGA CTG TAA CCA CG-3'). This CSW SELEX file was converted from tabular to FASTA format again. The top forty most abundant sequences were run through a Clustal W and phylogenetic tree analysis along with the most abundant sequences from cell SELEX pools developed in our laboratory for other cell membrane receptors. LIFR CSW aptamer candidates were chosen based on random sequence motif, abundance and distinction from other membrane receptor SELEX pools in the combined phylogenetic tree to eliminate non-selective binders for LIFR.

Three DNA aptamer candidate sequences were ordered conjugated to a biotin label CS6281: 5'-Biotin-
CTCCTCTGACTGTAACCACGGTGTACGTGACCCCATACGCACTCTAGCCACATACGTTCGCC
CACGCCTGTCGCTCGCGGGCATAGGTAGTCCAGAAGCC-3, CS4738: 5'-Biotin-CTCCTCTGAC
TGTAACCACGCGAGTCGCCTACGTACGCACACTTACCGCGCACGTCTGGGCAGGCGTGTCCC
GTGCATGCGCATAGGTAGTCCAGAAGCC-3'and CS70: 5'-Biotin-CTCCTCTGACTGTAACCA
CGGCGTACGTGACCCCATACGCACTCTAGCCACATACGTTCGCCCACGCCTGTCGCTCGCGG
GCATAGGTAGTCCAGAAGCC-'3.The LIFR CS70 aptamer candidate sequence was also ordered with a 5'-Cy5 fluorophore label. Two aptamer sequences previously developed in our laboratory to selectively bind LIFR expressing cells were used as positive controls for flow cytometry screening of cold switchable aptamer candidates. These positive control aptamers were LIF5-Cy5: 5'- Cy5-CTCCTCTG
ACTGTAACCACGGGCATAGGCGGGTGTGTATCTGCCAAGCGCGTGCTTGCTGATTCTCGCGC
GAATCACAGGCGCATAGGTAGTCCAGAAGCC-3' and LIF6-Biotin: 5'-Biotin- CTCCTCTGACTG
TAACCACGGTGCGTATGGACACGTCTGTACTGAGTGCGCATGTTGAGACGCATGCGTCGTGC
GTGTGTGCATAGGTAGTCCAGAAGCC-3'. For both positive control aptamers, binding to LIFR-

HEK cells is saturated at 200 nM, which is the concentration used for flow cytometry aptamer binding assays in this study. All DNA aptamer candidates were synthesized by Integrated DNA technologies. The LIFR CSW DNA aptamer candidates were dissolved with the required volume of PBS with Ca^{2+}/Mg^{2+} to make a 100 µM stock solution. Aptamer candidates were stored at 4°C.

2.9 LIFR CSW aptamer candidate flow cytometry binding and release assays

LIFR CSW DNA aptamer candidates were characterized by flow cytometry for selective binding to LIFR-HEK cells and subsequent release from these cells at 4°C. For the CS70 aptamer candidate which was directly conjugated to a Cy5 fluorophore, flow cytometry binding assay samples contained $1x10^6$ cells/mL and 200 nM of aptamer or Cy5 labeled DNA library in a 200 µL final volume of PBS with Ca^{2+}/Mg^{2+}. CS70 CSW aptamer, DNA library and LIF5 were each incubated with a LIFR-HEK cell sample and a HEK cell sample at 37°C for 60 min in the dark. A non-stained control containing 200,000 cells in PBS with Ca^{2+}/Mg^{2+} solution incubated without aptamers was prepared for both LIFR-HEK and HEK cell lines. Following incubation of the cell samples in the binding assay, the samples were pelleted by centrifugation at 200 x g and 22°C for 3 min. Each cell pellet was washed and resuspended in 400 µL PBS with Ca^{2+}/Mg^{2+} for flow cytometry analysis. To identify the dead cell population by flow cytometry, a LIFR-HEK and a HEK cell sample each containing 200,000 cells were run through the binding assay without aptamers and were stained for flow cytometry analysis with propidium iodide (PI) (Life Technologies) according to the manufacturer's instruction.

For the three biotinylated aptamer candidates CS70, CS6281 and CS4738 the flow cytometry binding assay was performed with the following modifications. Biotinylated aptamers (1 µM) were incubated with 20 µg/mL of Cy5 conjugated streptavidin (Life Technologies) in PBS with Ca^{2+}/Mg^{2+} for 30 min in the dark. Half of this biotinylated aptamer – Cy5 streptavidin complex solution was added to a $1x10^6$ cells/mL LIFR-HEK cell sample and the other half to a $1x10^6$ cells/mL HEK cell sample. The samples were made up to a 200 µL volume in PBS with Ca^{2+}/Mg^{2+}. Each sample contained 500 nM of biotinylated aptamer, 10 µg/mL of Cy5-Streptavidin (a 1:200 dilution of stock) and 200,000 cells in PBS with Ca^{2+}/Mg^{2+} buffer. A control to assess non-selective binding of fluorophore-labelled streptavidin to

cells in the absence of aptamer was prepared by combining 200,000 LIFR-HEK cells or HEK cells and 10 µg/mL of Cy5 conjugated streptavidin in 200 µL PBS with Ca^{2+}/Mg^{2+} buffer. The samples were incubated at 37°C for 60 min in the dark. Finally, samples were washed and resuspended in 400 µL of PBS with Ca^{2+}/Mg^{2+} for flow cytometry analysis.

To assess the ability of CSW SELEX developed aptamer candidates to release LIFR expressing target cells at 4°C a flow cytometry release assay was performed. The preparation of cell samples and controls was the same as previously described for the flow cytometry aptamer binding assay with one critical modification. The cell samples were prepared with $1x10^6$ cells/mL and 200 nM of aptamer made up to a final volume of 400 µL in PBS with Ca^{2+}/Mg^{2+} (400,000 cells each). Following the aptamer binding incubation at 37°C and washing of the cell pellets, the sample was divided into two 200-µL aliquots. The first 200-µL aliquot containing 200,000 cells with bound aptamer was made up to 400 µL in PBS with Ca^{2+}/Mg^{2+} for flow cytometry analysis. The second 200-µL aliquot of the aptamer 37°C binding sample was incubated on ice for 30 min and was termed the ice release sample. Following incubation, the ice release sample cells were pelleted at 200 x g and 4°C for 3 min and resuspended in 400 µL of PBS with Ca^{2+}/Mg^{2+}. An additional release assay was performed where the incubation time of the cell suspensions on ice was extended to 60 min.

Flow cytometry analysis of samples from both binding and release assays was performed using a Gallios flow cytometer. For each sample, 10,000 events within a polygon gate set on the forward and side scatter properties of the live HEK cell population were collected (Gate A, Figure 6B). Cy5 fluorescence of each sample was measured in the FL6 filter using the 633 nm laser for excitation, while PI stained samples were detected in the FL3 filter using the 488 nm laser for excitation.

2.10 Flow cytometry binding and release assay data analysis and statistics

Analysis of the flow cytometry screening of CSW aptamer candidate binding and release from LIFR-HEK target cells was performed using Kaluza analysis software (Beckman Coulter). The live cell population gate was set using the PI stained LIFR-HEK cell sample. Dead cells were detected and gated on a highly PI fluorescence population in a one parameter histogram for PI fluorescence (Gate B, Figure

6A). This dead cell gate was backgated onto the log side scatter (SSC) versus forward scatter (FSC) height dot plot of this LIFR-HEK cell sample (Figure 6B, green population). The live cell gate was then set to eliminate these highly PI fluorescent dead cells from the main LIFR-HEK cell population in the log SSC versus FSC dot plot (Figure 6B, C). This live cell gate was then applied to all cell samples in a given analysis (Figure 6D, E).

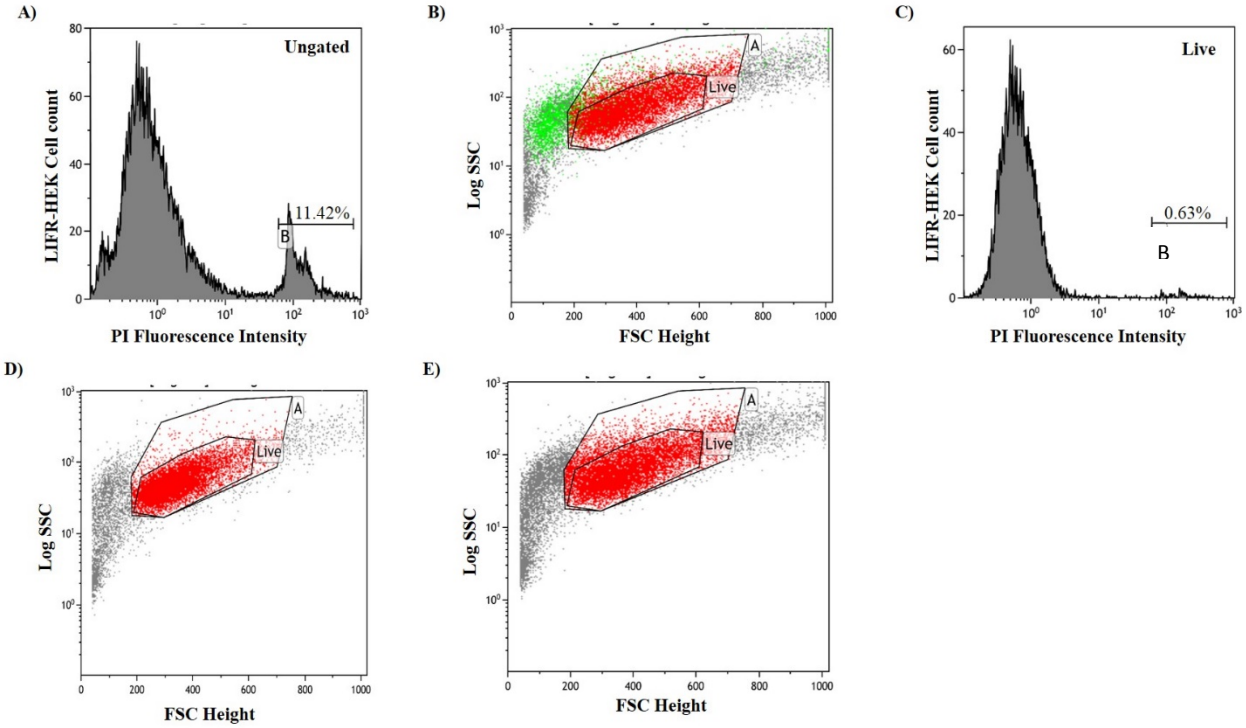


Figure 6. Live cell gating strategy for flow cytometry aptamer binding and release assays. A) PI fluorescence emission distribution of an LIFR expressing HEK (LIFR-HEK) cell sample containing 200,000 cells stained with propidium iodide (PI) for 30 min in the dark was analyzed by flow cytometry. The second highly fluorescent population of cells was gated B and represents the dead cell population. The cell sample contained 11.42% dead cells. B) Back gating of gate B (green) set on the highly PI fluorescent population of LIFR-HEK cells onto a log side scatter (SSC) versus forward scatter (FSC) height dot plot. Therefore the dead cell population is separated from the main live cell population by higher granularity and small size. Gate A (red) was roughly set in the flow cytometry protocol to eliminate debris and doublets in order to collect 10,000 events in the estimated live cell population. The gate marked live was refined post collection from gate A to remove highly PI fluorescent dead cells from further analysis. C) Live gated LIFR-HEK sample stained with PI showing a reduced high intensity PI fluorescence peak, and therefore a decrease in dead cells compared to the ungated population (0.63% compared to 11.42% in panel A). D) and E)The live cell gate applied to a HEK cell sample and LIFR-HEK cell sample, respectively, stained with CS70-Cy5 aptamer candidate. The Cy5 fluorescence intensity distribution of this live cell population was analyzed for aptamer binding.

Cy5 fluorescence intensity distribution of the live cell population of each cell sample in the binding and release assays was displayed in a one parameter histogram of count versus Cy5 log fluorescence intensity. The median Cy5 fluorescence intensity from the live cell population of samples incubated with aptamer candidates and binding controls was corrected for cell autofluorescence using the Cy5 median fluorescence intensity of the corresponding non-stained cell sample. Cy5 median fluorescence intensity therefore was proportional to aptamer binding. Percent release due to incubation on ice was calculated as: (MFI Binding-MFI Release)/MFI Binding x 100, where MFI binding is the median Cy5 fluorescence intensity of the cell sample incubated with aptamer at 37°C and MFI release indicates the median Cy5 fluorescence intensity of an aliquot of the same cell sample following incubation on ice. Therefore the percent release indicates the percentage of aptamers bound to the LIFR-HEK or HEK cells at 37°C that were released from these cells during incubation on ice.

For the flow cytometry binding assay using CS70-Cy5 labeled aptamer candidate a two way Type 1 ANOVA (factorial, balanced) using cell type, LIFR-HEK or HEK, and aptamer, CS70, LIF5 or DNA library, as variables was performed using autofluorescence corrected Cy5 median fluorescence intensity data. The assay was performed in triplicate. For a significant F-statistic, T-tests were performed and corrected for multiple comparisons using Bonferroni-Holm correction with a significance threshold of $p<0.05$ and $n = 3$. Statistical analysis was performed using the R software program (see Appendix 1 for script of R commands and software output). Graphs and fold change in Cy5 median fluorescence intensity were generated and computed in Excel 2013.

3. RESULTS

3.1 Confirmation of LIFR expression in LIFR-HEK cell line

The development and screening of cold switchable aptamers that selectively bind LIFR at 37°C relied on a functional *in vitro* system in which a HEK cell line had been established to overexpress LIFR. An antibody based assay was used to determine the level of LIFR expression in LIFR-HEK and HEK control cell lines, in which anti-LIFR AF700 antibody staining was detected by flow cytometry. Comparing the median AF700 fluorescence intensity of LIFR-HEK and HEK anti-LIFR stained cell samples, each corrected for the autofluorescence contribution of the respective cells alone, LIFR-HEK cells exhibited 43-fold greater median fluorescence intensity and therefore LIFR expression compared to HEK cells (Figure 7). Confirmation of LIFR overexpression in the LIFR-HEK cell line validated the use of this cell line for positive selection of cold switchable aptamers, while the negligible expression of LIFR in the HEK control cell line confirmed the use of this cell line for negative selection.

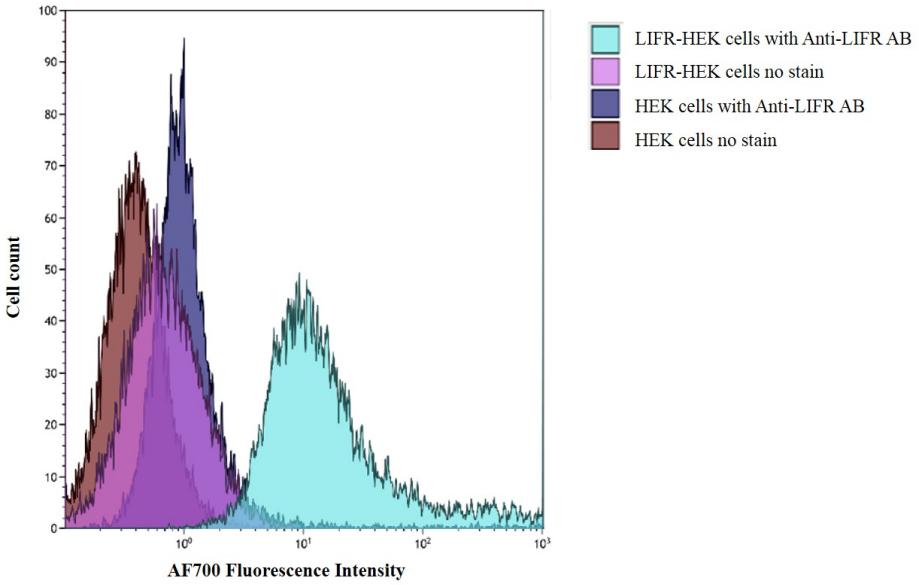


Figure 7. Flow cytometry analysis of LIFR expression in LIFR expressing HEK and HEK cell lines. Cell samples containing $1x10^6$ cells/mL were incubated with 5 µg/mL of AF700 conjugated mouse anti-human LIFR monoclonal antibody in PBS with Ca^{2+}/Mg^{2+} buffer for 60 min at 4°C in the dark. AF700 fluorescence intensity of antibody stained and non-stained LIFR expressing HEK (LIFR-HEK) and HEK cell samples was measured by flow cytometry. Non-stained controls were run to identify the level of cell autofluorescence in this assay. AF700 fluorescence histograms for each cell sample represent events measured for a sample within the live cell gate.

3.2 Selection of cold switchable aptamers targeting LIFR with CSW SELEX procedure

From each round of CSW SELEX potential DNA aptamer candidates were collected from a supernatant that contained only those molecules that selectively bound LIFR at 37°C and released LIFR expressing cells after 30 min incubation on ice. This supernatant was run through agarose gel electrophoresis to separate potential DNA aptamer candidates from fragmented chromosomal DNA and cellular debris. In Figure 8A the recovered CSW SELEX supernatant contains a DNA band running a distance equal to that of the DNA ladder 100 bp band and a positive control band which contains a PCR amplified SELEX pool. The 100 bp DNA band from the CSW SELEX lane was extracted and purified to isolate a CSW SELEX pool that contains potential cold switchable aptamer candidates selected for during CSW SELEX. The CSW SELEX pool was PCR amplified in preparation for another round of selection. In Figure 8B an example PCR reaction result is shown in which the CSW SELEX pool PCR product exhibits two DNA bands identical to those seen in the positive control from the previously PCR amplified SELEX pool. The top 100 bp band contains potential DNA aptamer candidates and the bottom 80 bp band is the primer dimers. The negative PCR control run without template shows only the 80 bp primer dimer band and therefore there is no sign of DNA contamination.

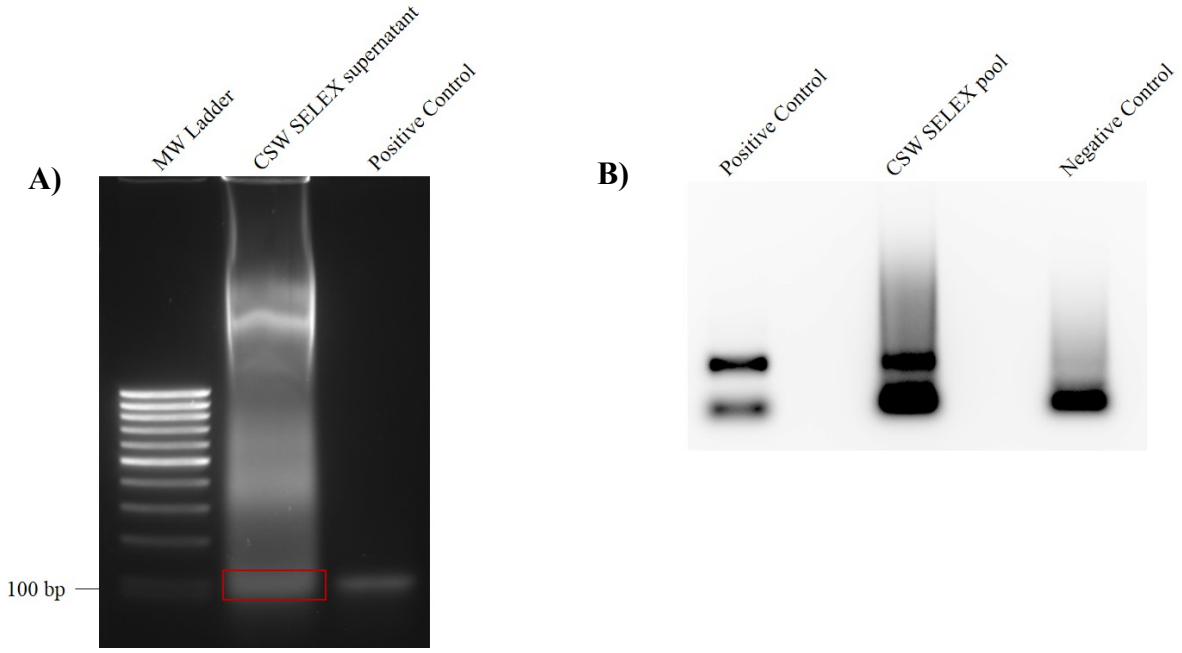


Figure 8. Cold switchable SELEX DNA pool purification and PCR amplification. The supernatant recovered from the cold switchable (CSW) SELEX procedure contained the potential DNA aptamer candidates evolved to selectively bind LIFR expressing cells at 37°C and release these target cells after incubation on ice. A) The molecular weight DNA ladder (lane 1), recovered supernatant from CSW SELEX (lane 2) and a purified SELEX pool PCR product as a positive control (lane 3) were run on a 1% agarose gel for 45 min at 160V. The gel was visualized by UV transillumination and the 100 bp gel band from the CSW SELEX lane containing potential DNA aptamer candidates, as indicated by the red rectangular inset, was removed and purified. B) The purified CSW SELEX pool of DNA molecules was then amplified by PCR. The positive control containing a previously amplified SELEX pool PCR product (lane 1), CSW SELEX pool PCR product (lane 2) and the negative control PCR reaction that contained no DNA template (lane 3) were run on a 3% agarose gel and visualized by Cy5 fluorescence emission.

Six rounds of CSW SELEX were performed beginning with the twelfth round pool of DNA molecules evolved using cell SELEX to be selective binders for LIFR expressing cells. These six rounds of CSW SELEX included four rounds with only positive selection for LIFR-HEK cell binding at 37°C and release of these target cells after incubation on ice, followed by two rounds that included positive selection, release on ice and negative selection to remove DNA molecules which bound to HEK untransduced cell line. The PCR amplified CSW SELEX sixth round (CSW6) pool of Cy5 labeled DNA molecules was then tested using flow cytometry for their ability to selectively bind LIFR at 37°C and release LIFR expressing cells after incubation on ice. The cell autofluorescence corrected median Cy5

fluorescence intensity of LIFR-HEK cells incubated with CSW6 pool at 37°C was 3-fold greater than that of LIFR-HEK cells incubated with DNA library (Figure 8A). This result indicates that 50% of the DNA molecules in the CSW6 pool exhibit at least 3-fold greater binding to LIFR-HEK cells than a randomly generated pool of DNA sequences. Therefore the selective pressure created by positive selection with LIFR-HEK cells has successfully enriched for DNA molecules with the capacity to bind LIFR-HEK cell line at 37°C. The selectivity of the CSW6 pool was analyzed by comparing the autofluorescence corrected median Cy5 fluorescence intensity between LIFR-HEK and HEK cells incubated with CSW6 pool at 37°C. The median fluorescence intensity of LIFR-HEK cells was 2-fold greater than that of HEK cells (Figure 9A). This finding provides evidence that the CSW6 pool contains DNA molecules which are selective LIFR binders.

The ability of DNA molecules in the CSW6 pool to release bound LIFR-HEK cells following incubation on ice was analyzed by taking an aliquot of a cell suspension that had been incubated with the CSW6 pool at 37°C and cooling it on ice for 30 min. Following incubation on ice, there was an 85% release of CSW6 round pool DNA molecules that bound to LIFR-HEK cells at 37°C. This was based on the 7-fold decrease in autofluorescence corrected Cy5 median fluorescence intensity of LIFR-HEK cells stained with the CSW6 round pool observed when these cells were cooled from 37 C to 4°C though ice incubation (Figure 9A). To determine the percentage of LIFR-HEK cells that were completely released from any bound CSW6 DNA molecules following incubation on ice, the percentage of LIFR-HEK cells in a negative gate based on cell autofluorescence was analyzed (Figure 9B). After 60 min incubation with the CSW6 pool at 37°C, 67.0% of LIFR-HEK cells had Cy5 fluorescence emission above that of cell autofluorescence indicating that the majority of the target cells had been bound by DNA molecules from the CSW6 pool. In contrast, in an aliquot of this cell suspension which was subsequently incubated on ice, only 15.4 % percent of LIFR-HEK cells had fluorescence emission above the range of cell autofluorescence. Therefore, 51.6% of LIFR-HEK cells that had been bound by CSW6 DNA molecules at 37°C were released from these DNA molecules after incubation on ice.

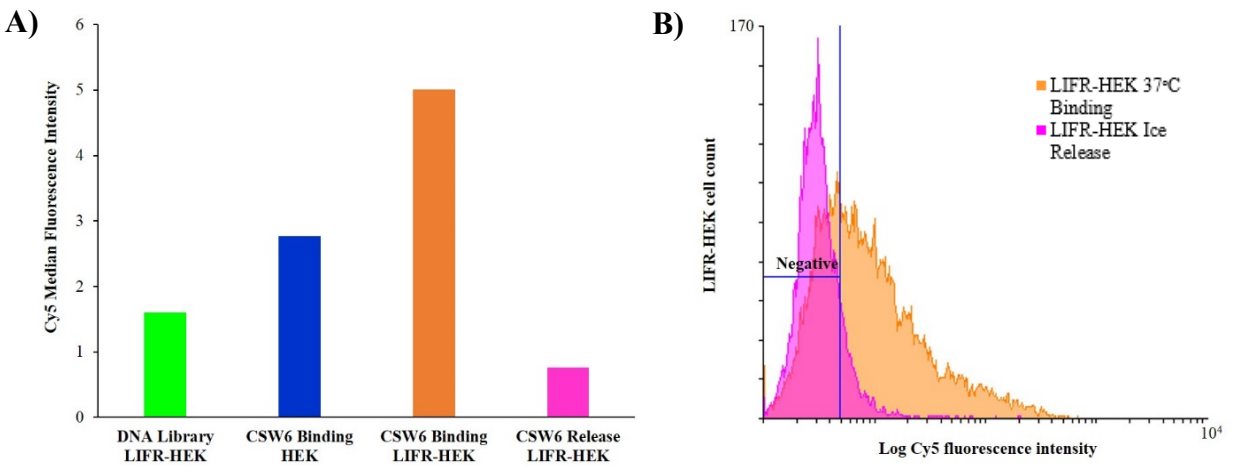


Figure 9. Flow cytometry analysis of the sixth round CSW SELEX pool for selective binding of LIFR expressing HEK cells at 37°C and subsequent release of the cells after incubation on ice. A) Cell samples for flow cytometry were prepared with $1x10^6$ cells/mL from the LIFR expressing HEK (LIFR-HEK) cell line or HEK cell line and 200 nM of CSW SELEX sixth round pool DNA (CSW6) or DNA library. Cell samples containing Cy5 labeled DNA molecules were incubated for 60 min at 37°C in the dark. The release sample was an aliquot of a LIFR-HEK cell sample stained with CSW6 pool at 37°C that was subsequently incubated on ice for 30 min. Median Cy5 fluorescence intensity (Cy5 MFI) of the live cell population of each cell sample was corrected for cell autofluorescence by subtracting the Cy5 MFI of a non-stained cells alone control. B) Cy5 fluorescence emission distribution of LIFR-HEK cells stained with CSW6 pool DNA molecules at 37°C before and after 30 minutes incubation on ice. The negative gate spans the spectrum of LIFR-HEK cell autofluorescence set by a non-stained control, indicating no Cy5 labeled DNA bound to LIFR-HEK cells. The CSW6 binding sample at 37°C has 33.0% of LIFR-HEK cells within the negative gate while the CSW6 release sample has 84.6% of LIFR-HEK cells within the negative gate.

3.3 Selection of DNA aptamer candidates from the sequenced CSW SELEX sixth round pool.

The LIFR CSW SELEX sixth round (CSW6) pool contained 28,890 sequenced DNA molecules that were identified by the unique forward primer and reverse primer sequences of the Havard library, as well as the TGACTGAC barcode specific to CSW6 DNA molecules. These DNA molecules represent 6,567 unique sequences each of which is a potential cold-switchable (CSW) aptamer candidate. The 60 bp random region from each of the top forty most abundant sequences in the CSW6 pool were run through a Clustal W alignment analysis to group these sequences into families based on common motifs. The alignment produced two families of aptamer candidates for which the shared random sequence motif identity was over 95% within each family and less than 60% between families (Figure 10A).

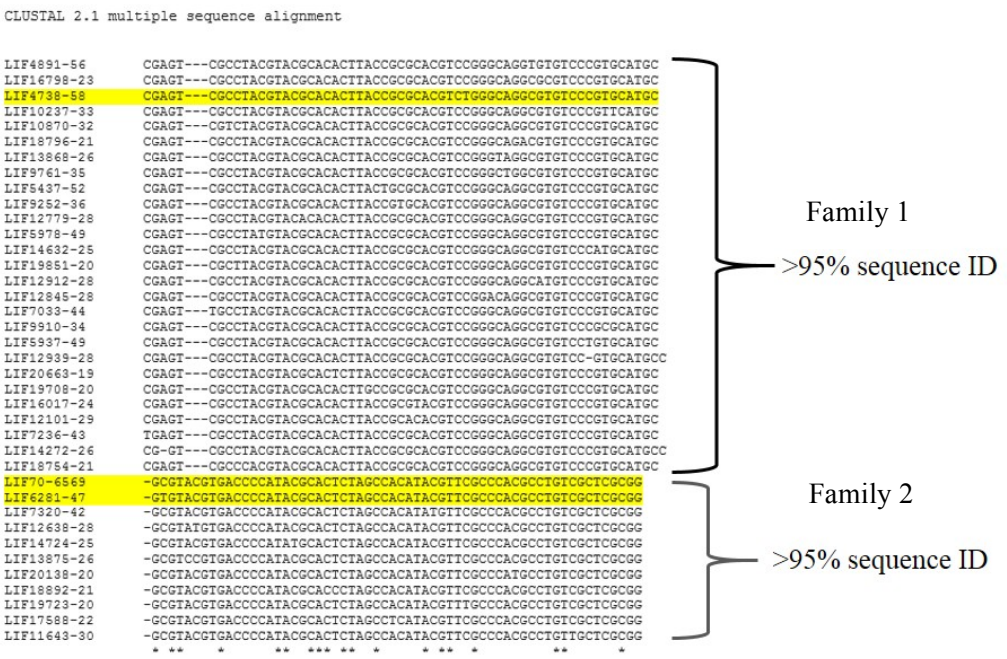
To select aptamer candidate sequences that were selective for LIFR expressing cells, the entire 100 bp sequence from each of the top 40 most abundant CSW6 pool DNA molecules were run in a phylogenetic tree analysis along with the top 40 sequences from other cell SELEX pools selected for different cell membrane receptors. As all cell SELEX procedures in our laboratory use a HEK cell *in vitro* expression system, sequences from SELEX pools evolved to bind different receptors that are closely related in a phylogenetic tree analysis most likely bind to a common component of the HEK cell membrane and not the recombinant receptor target of interest. The LIFR CSW aptamer candidates were present in two main clusters of the SELEX pool phylogenetic tree, which was indicative of the two main families based on sequence identity (Figure 10B). There were a few surface plasminogen 1 (S1P) receptor aptamer candidate sequences present in the LIFR clusters, however they were in the minority. The most abundant aptamer candidate was chosen from each of the LIFR CSW random sequence motif families, specifically LIF70-6569 and LIF4738-58, where these sequences were present in the CSW6 pool 6569 and 58 times, respectively. Abundance of an aptamer candidate in the CSW6 pool reflects selective pressure during the CSW SELEX process. An additional sequence, LIF6281-47 found 47 times in the CSW6 pool, was chosen from random sequence family 2 based on phylogenetic distance within the LIFR CSW6 pool cluster. Even though it is 95% identical in sequence to LIF70-6569, the change of even one nucleotide in an aptamer sequence can have an impact on secondary structure and therefore LIFR target binding and release.

The three aptamer candidates ordered were abbreviated CS70, CS6281 and CS4738 (sequences LIF70-6569, LIF6281-47 and LIF4738-58, respectively). The lowest potential energy structures for each 100 bp aptamer candidate at both 37°C and 4°C were generated by m-fold software (Figure 11A-C). All three aptamer candidates form lowest energy secondary structures with a large open loop motif and four small stem loop motifs at 37°C. The similarity in structure indicates a possible secondary structure requirement for LIFR binding. At 4°C the predicted secondary structures of each aptamer candidate exhibited more complex internal base pairing and stem loop motifs that were not stable at 37°C. This

provides theoretical evidence for how the CSW aptamer secondary structure could change substantially to disrupt LIFR target binding and release LIFR expressing cells following incubation on ice.

The three aptamer candidates were ordered in conjugate forms that allowed detection of aptamer binding and release from target LIFR-HEK cells by flow cytometry screening assays. CS70 was ordered in two forms, directly conjugated to a Cy5 fluorophore and conjugated to biotin. CS6281 and CS4738 were ordered conjugated to biotin. For the biotinylated aptamers flow cytometry detection of binding was accomplished by Cy5 conjugated streptavidin detection agent. The biotinylated aptamer candidates were ordered as they can be used in both flow cytometry screening procedures and, if the desired activity is found, directly in a streptavidin coated magnetic bead based cell isolation assay.

A)



B)

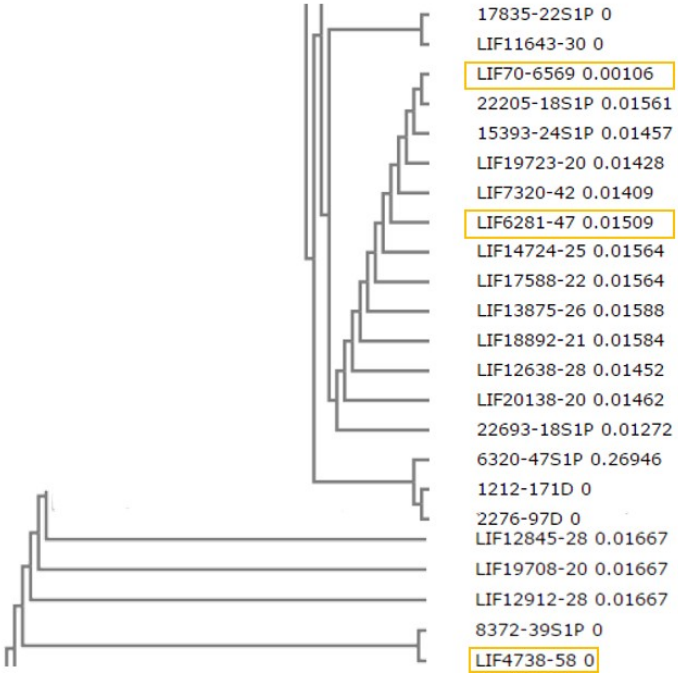


Figure 10. Selection of aptamer candidate sequences from the CSW SELEX sixth round pool. A) Clustal W alignment of the 60 bp random regions for the top 40 most abundant DNA sequences in the cold switchable SELEX sixth round (CSW6) pool. Two families of sequences emerge that have 95% sequence identity within each family. Common nucleotides in all families are denoted with an asterisk. The three sequences highlighted in yellow were ordered for screening as LIFR cold-switchable aptamer candidates. B) Clustal W phylogenetic tree analysis of the top 40, 100 bp DNA sequences from eight

different cell SELEX procedures using the HEK *in vitro* expression system. Only LIFR CSW6 sequence clusters are shown. Ordered aptamer candidates are highlighted in yellow rectangles. Receptor targets of aptamer sequences denoted LIF for leukemia inhibitory factor and S1P for surface plasminogen 1.

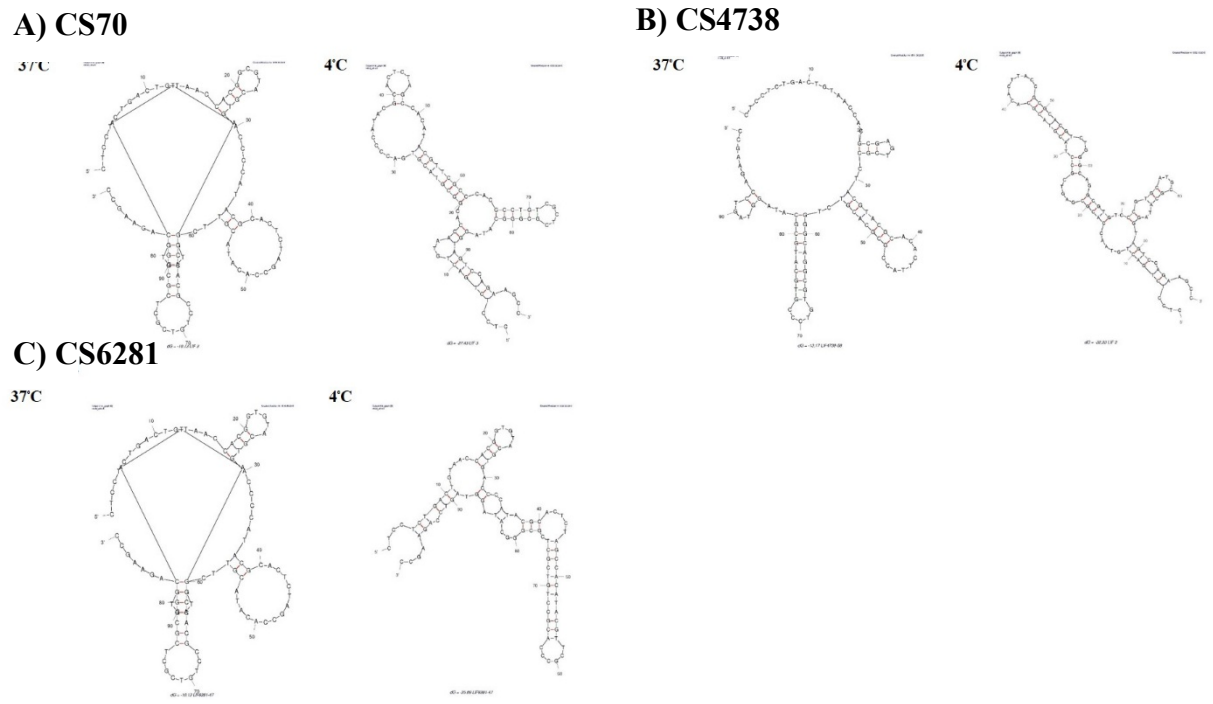


Figure 11. Predicted secondary structures for the three cold switchable LIFR aptamer candidates that were chosen from the sixth round CSW SELEX pool for further analysis. Lowest potential energy structures for three cold switchable aptamer candidates evolved to bind LIFR expressing cells at 37°C and release them following incubation on ice. Secondary structures were predicted by m-fold software at both 37°C and 4°C.

3.4 Flow cytometry screening of CS70-Cy5 labeled aptamer candidate.

A flow cytometry binding assay was performed to assess the degree of binding to LIFR-HEK and HEK cell lines of three different Cy5 labeled DNA affinity ligands: CS70 aptamer candidate, LIF5 positive control aptamer for selective LIFR binding and the randomly generated Havard DNA library sequences (Figure 12A). The assay was performed in triplicate, and the autofluorescence corrected median Cy5 fluorescence values for each sample were analyzed by a two-way Type 1 ANOVA analysis. There was a significant ($p < 0.05$) effect of DNA affinity ligand on Cy5 median fluorescence intensity

($p = 6.659x10^{-9}$), while the effect of cell line on Cy5 median fluorescence intensity ($p = 0.09664$) was not different. There was a significant ($p < 0.05$) interaction between DNA affinity ligand and cell line effects on the Cy5 median fluorescence intensity ($p = 0.03462$) meaning that the binding affinity for a particular cell line differs between DNA affinity ligands and DNA affinity ligands differ in binding selectivity for LIFR-HEK or HEK cell lines. To examine these differences further pairwise t-tests were performed to assess differences in DNA affinity ligand binding to the LIFR-HEK cell line, and the selectivity of CS70 and LIF5 aptamers for LIFR-HEK cells compared to the control HEK cell line in which LIFR target is not expressed.

The CS70 aptamer candidate exhibited a significant ($p < 0.05$) 15-fold increase in LIFR-HEK cell median Cy5 fluorescence intensity compared to DNA library at 37°C ($p = 2.4x10^{-6}$, n = 3, Bonferroni-Holm correction) (Figure 12C). Therefore, the CS70 aptamer sequence binds LIFR-HEK target cells at 37°C greater than the original randomly generated pool of nucleic acids from which it was selected. Thus the positive selection component of CSW SELEX for LIFR-HEK target cells at 37°C appears to have been successful for this aptamer candidate. There was no difference in Cy5 median fluorescence intensity between LIFR-HEK and HEK cell samples incubated with CS70 aptamer candidate for 60 minutes at 37°C ($p = 0.99$, n = 3, Bonferroni-Holm correction) (Figure 12B). This is in contrast to the LIF5 aptamer, a positive control for selective binding of LIFR expressing cells at room temperature. LIF5 aptamer exhibits a significant ($p < 0.05$) 7-fold greater binding to LIFR-HEK cells compared to the DNA library ($p = 0.00424$, n = 3, Bonferroni-Holm correction) (Figure 12C), as well as a significant ($p < 0.05$) 3-fold greater binding to LIFR-HEK cell line compared to the HEK cell line ($p = 0.026$, n = 3, Bonferroni-Holm correction) Figure 12B). Therefore, unlike LIF5 aptamer, the CS70 aptamer candidate does not appear to selectively bind LIFR and most likely has been evolved to bind another component of the HEK cell membrane. This indicates that the negative selection component of CSW SELEX was not effective in removing this non-selective binder.

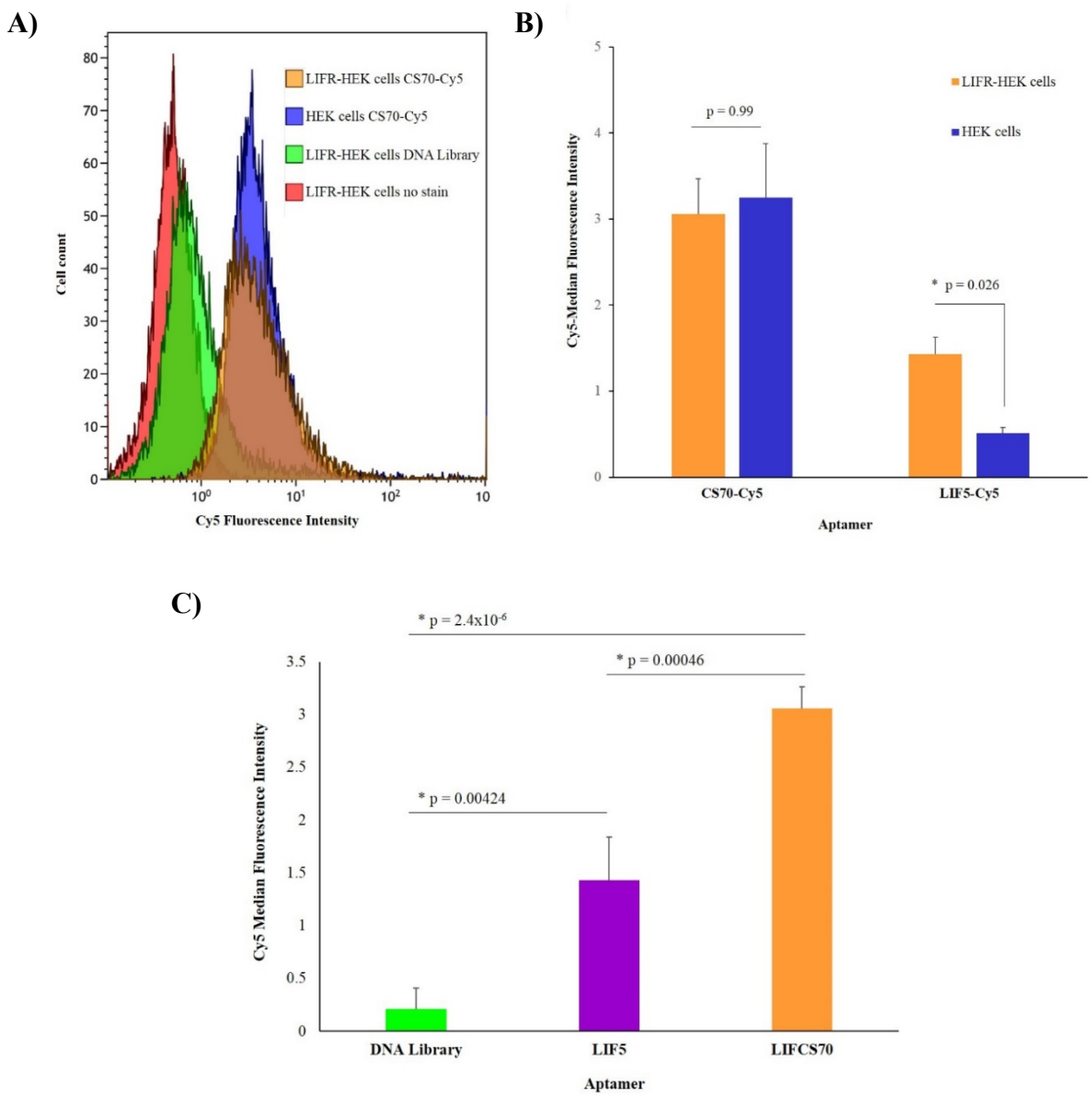


Figure 12. Flow cytometry screening of CS70 cold switchable aptamer candidate for selective binding to LIFR expressing HEK cells. A) Cell samples were prepared for the flow cytometry aptamer binding assay with $1x10^6$ cells/mL and 200 nM of DNA affinity ligand. Samples were incubated for 60 min at 37°C in the dark. Cy5 fluorescence emission distributions of the live cell population are presented for the indicated cell samples. B) Comparison of CS70 aptamer candidate and LIF5 aptamer binding to LIFR expressing HEK (LIFR-HEK) and HEK cell lines. Cy5 median fluorescence intensity of each cell sample was corrected for cell autofluorescence from a non-stained cell sample and therefore representative of aptamer binding. C) Comparison of CS70 aptamer candidate, LIF5 aptamer and random sequence Havard DNA library binding to LIFR-HEK target cells. Cy5 median fluorescence intensity for each cell sample has been corrected for autofluorescence based on a non-stained LIFR-HEK cell sample. All p values presented are the result of pairwise t-tests following a two-way factorial ANOVA with DNA affinity ligand and cell type variables. The design was balanced with n = 3 for all samples and p values were corrected for multiple comparisons using the Bonferroni-Holm correction. Asterisks indicate significance evaluated at threshold $p < 0.05$.

The ability of CS70-Cy5 aptamer candidate to release from LIFR-HEK cells following incubation on ice was analyzed by a flow cytometry release assay. An aliquot of the LIFR-HEK cell sample incubated with CS70 aptamer at 37°C was incubated on ice to measure the change in Cy5 median fluorescence intensity and therefore aptamer release due to cooling on ice. No shift in Cy5 fluorescence intensity distribution of CS70-Cy5 stained LIFR-HEK cells was seen following 30 min incubation on ice (data not shown). However, increasing the incubation time to 60 min the Cy5 fluorescence distribution of CS70 stained LIFR-HEK cells shifted to a lower intensity, moving towards that of LIFR-HEK cells alone sample (Figure 13A). A similar shift in Cy5 fluorescence emission intensity was observed with CS70 stained HEK cells after 60 min incubation on ice, as CS70 binds non-selectively it was expected that any release would also not be selective for LIFR expressing cells (Figure 13B). There was an average 1.5-fold (n = 2) decrease in autofluorescence corrected median Cy5 fluorescence intensity between the CS70-Cy5 stained LIFR-HEK cell sample before and after incubation on ice for 60 min (Figure 13C). The same decrease in Cy5 median fluorescence intensity and therefore aptamer binding was observed comparing CS70 stained HEK cells at 37°C and an aliquot of these cells incubated on ice (Figure 13C). Therefore, there was a 35% decrease in Cy5 median fluorescence intensity due to CS70 aptamer release after LIFR-HEK and HEK cells with bound CS70 were incubated on ice for 60 min, indicating non-selective cold-switching activity.

An interesting result from the flow cytometry release assays was that LIFR-HEK cells stained with LIF5 aptamer at room temperature exhibited a 2.7-fold decrease in Cy5 median fluorescence intensity after a 60 min incubation on ice compared to cells not incubated on ice (Figure 14). LIF5 aptamer is a selective binder for LIFR expressing cells and was evolved by traditional cell SELEX. Therefore this apparent cold switchable activity of this aptamer was not purposely selected for during *in vitro* evolution.

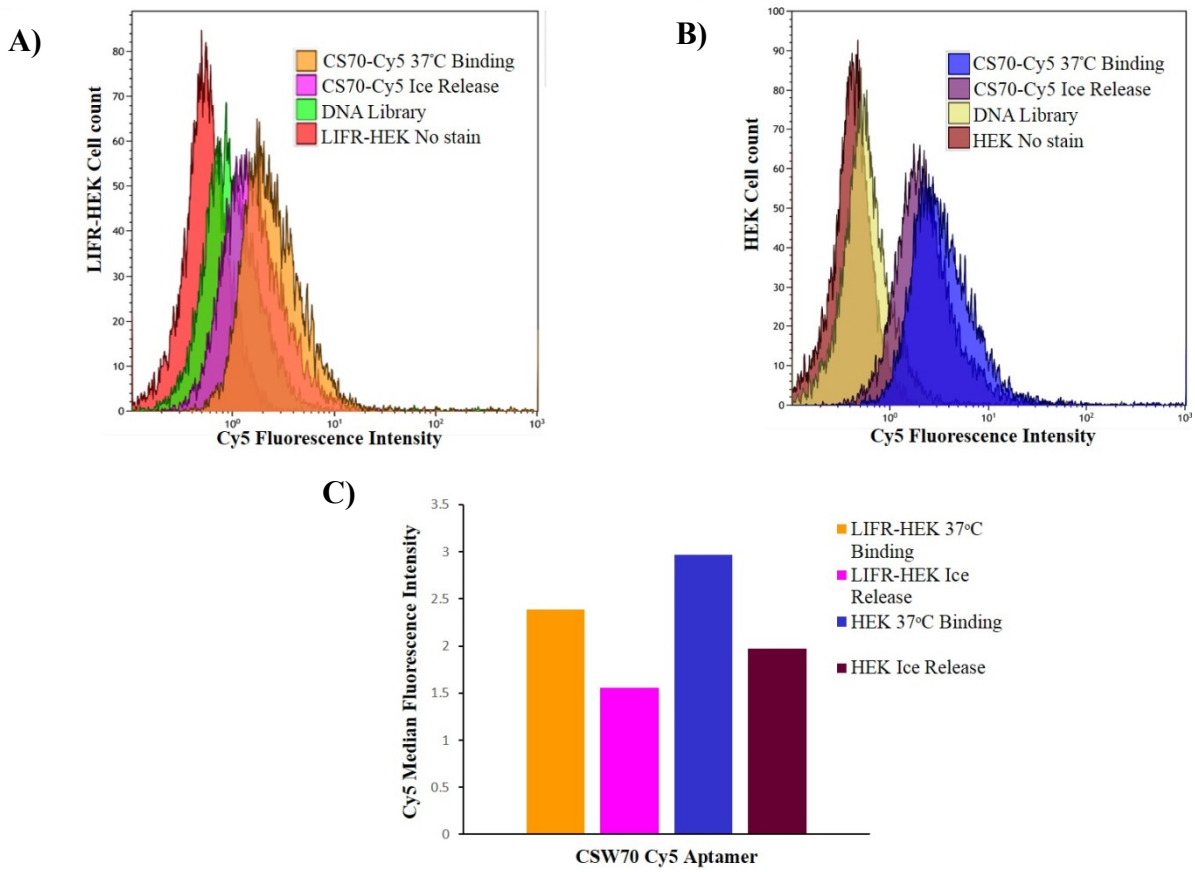


Figure 13. Cold-dependent release of CS70-Cy5 aptamer candidate from LIFR expressing HEK and HEK cell lines. A) Ice dependent release of CS70 aptamer candidate from LIFR expressing HEK (LIFR-HEK) target cells. LIFR-HEK cell samples were prepared with $1x10^6$ cells/mL and 200 nM of Cy5 labeled DNA library, CS70-Cy5 aptamer or a no aptamer stain control for cell autofluorescence. Following incubation of cell samples for 60 min at 37°C in the dark, an aliquot of the CS70-Cy5 37°C binding sample was incubated on ice for 60 min and was termed the CS70-Cy5 ice release sample. Cy5 fluroescence intensity distributions for each cell sample are shown for the live gated cell population. B) Ice dependent release of CS70 aptamer candidate from HEK control cells. As CS70 aptamer candidate is not selective for LIFR expressing cells an identical flow cytometry release assay was performed as in A with the untransduced HEK cell line. C) The average autofluroescence corrected Cy5 median fluorescence intensity for LIFR-HEK and HEK cells stained with CS70-Cy5 aptamer candiate before and after 60 min incubation on ice (n =2). There was a 1.5-fold decrease or 35% reduction in CS70-dependent Cy5 median flourescence intensity of both cell types due to incubation on ice.

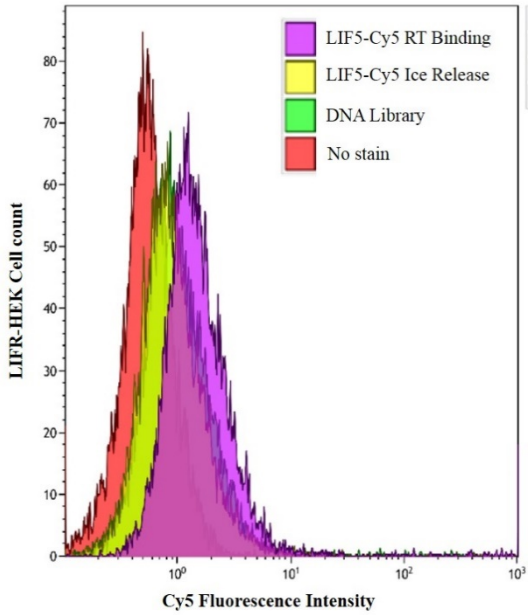


Figure 14. Cold-dependent release of LIF5 aptamer from LIFR expressing HEK cell line. LIF5-Cy5 aptamer is a selective binder of LIFR expressing cells that was developed by a traditional cell SELEX procedure and was used in this study as a positive control for LIFR selective binding. LIFR expressing HEK (LIFR-HEK) cell samples were prepared with $1x10^6$ cells/mL and 200 nM of Cy5 labeled DNA library, LIF5-Cy5 aptamer or a no aptamer stain control for cell autofluorescence. Following incubation of cell samples for 60 min at room temperature in the dark, an aliquot of the LIF5-Cy5 room temperature binding sample was incubated on ice for 60 min and was termed the CS70-Cy5 ice release sample. Cy5 fluroescence intensity distributions for each cell sample are shown for the live gated cell population. There was a 2.7-fold decrease or 64% reduction in LIF5-dependent Cy5 median flourescence intensity of LIFR-HEK cells due to incubation on ice.

3.5 Flow cytometry screening of three biotinylated cold switchable aptamer candidates.

To compare the affinity and selectivity of the three cold switchable aptamer candidates, CS70, CS6281 and CS4738, by flow cytometry the detection of these biotinylated aptamer candidates with Cy5 conjugated streptavidin needed to be optimized. The detection of CS70 biotinylated aptamer binding was more sensitive if the biotinylated aptamers were first incubated with Cy5-Streptavidin prior to incubating the aptamers with the LIFR-HEK cells. This procedure resulted 1.5-fold greater shift in Cy5 median fluorescence intensity and therefore resolution from cell autofluorescence, compared to staining with an equal concentration of Cy5 streptavidin post binding incubation of CS70 aptamer with LIFR-HEK cells (data not shown). However, the LIFR-HEK cells incubated with CS70 aptamer at 37°C showed a 2.7-fold decrease in Cy5 fluorescence intensity when detected by Cy5 streptavidin using 500 nM of the

biotinylated conjugate compared to using 200 nM of CS70-Cy5 aptamer directly in the same assay (Figure 15A vs 12A). However, in spite of the reduced sensitivity the shift in LIFR-HEK cell Cy5 median fluorescence intensity due to CS70 biotinylated aptamer can be resolved 2.8-fold from both cell autofluorescence and a Cy5-streptavidin stain alone cell sample, used to control for non-selective Cy5 streptavidin binding to LIFR-HEK cells (Figure 15A).

The three cold switchable aptamer candidates for LIFR expressing HEK cells did not show selective binding for LIFR as determined by a flow cytometry binding assay. The difference in background staining corrected Cy5 median fluorescence intensity between LIFR-HEK and HEK cells incubated at 37°C with biotinylated aptamer was 1-fold, 0.9-fold and 0.8-fold for CS70, CS6281 and CS4738, respectively (Figure 15B). Therefore, the cold switchable aptamer candidates bind with similar affinity to HEK cells when the LIFR target is not present. This is in contrast to the LIF6 biotinylated aptamer, a positive control for LIFR selective binding, which binds LIFR-HEK cells at room temperature with a 16.5-fold greater affinity than HEK cells (Figure 15B).

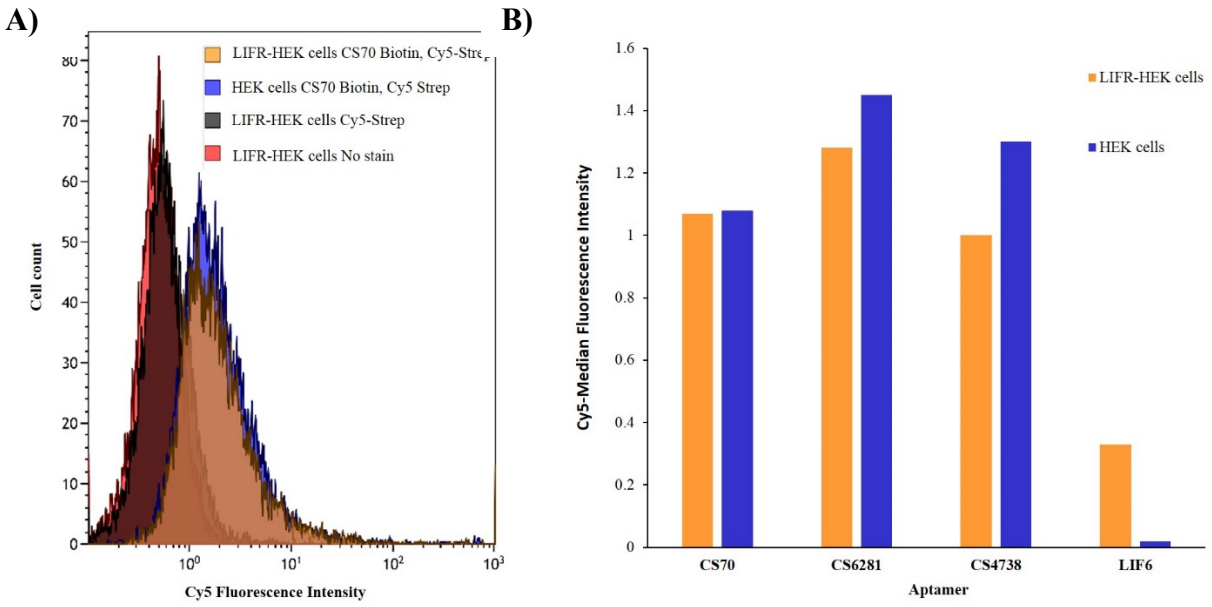


Figure 15. Screening of the selectivity of biotinylated cold switchable aptamer candidates for LIFR expressing HEK cells. A) Flow cytometry binding assay for CS70 biotinylated aptamer candidate detected with Cy5 labeled streptavidin (Cy5-Strep). LIFR expressing HEK (LIFR-HEK) and HEK cell samples were prepared for flow cytometry analysis by incubating $1x10^6$ cells/mL with 500 nM of CS70

biotinylated aptamer, pre-stained with 10 µg/mL of Cy5-Strep, for 60 min at 37°C in the dark. The Cy5-Strep control contained $1x10^6$ LIFR-HEK cells/mL and 10 µg/mL of Cy5 conjugated streptavidin. As a negative binding control for cell autofluorescence prepared a LIFR-HEK non-stained sample with $1x10^6$ LIFR-HEK cells/mL only. Cy5 fluroescence emission distribution histograms displayed for each cell sample represents the live cell population only. This same binding assay was repeated for CS6281 and CS4738 aptamer candidates as well as an LIFR selective biotinylated aptamer LIF6 (histograms not shown). B) Binding selectivity for LIFR of cold switchable aptamer candidates. Cy5 median fluroescence intensity values of LIFR-HEK and HEK cell samples incubated with each cold switchable aptamer candidate and LIF6 positive control were corrected for background Cy5-streptavidin staining by subtracting the Cy5 median fluorescence intensity of the Cy5-Strep control for the corresponding cell line (n = 1).

The ability of CS70, CS6281 and CS4738 biotinylated aptamer candidates to release LIFR-HEK and HEK cells following incubation on ice was determined using a flow cytometry release assay. To determine cooling dependent release an aliquot of LIFR-HEK cells incubated with Cy5-streptavidin stained cold switchable biotinylated aptamers at 37°C was incubated for 60 min on ice. For all three cold-switchable aptamer candidates there was a shift in biotinylated aptamer stained LIFR-HEK cell Cy5 fluorescence emission distribution to a lower intensity similar to cell autofluorescene due to incubation on ice (Figure 16A). A similar decrease in Cy5 median fluorescence intensity of HEK cells stained with each cold switchable aptamer candidate at 37°C was seen after incubation on ice for 60 min (Figure 16A). LIFR-HEK cells stained with biotinylated CS70 aptamer exhibited a 1.6-fold decrease or 38% reduction in Cy5 median fluorescence intensity following incubation on ice, while HEK cells stained with biotinylated CS70 aptamer showed a 1.5-fold or 33% decrease in Cy5 median fluorescence intensity after incubation on ice (Figure 16B). For CS6281 and CS4738 aptamer candidates the decrease in Cy5 median fluorescence intensity between samples incubated on ice and not incubated on ice was 2-fold (50%) and 1.5-fold (26%) for LIFR-HEK cells and 1.4-fold (33%) and 1.5-fold (33%) for HEK samples, respectively (Figure 16B).

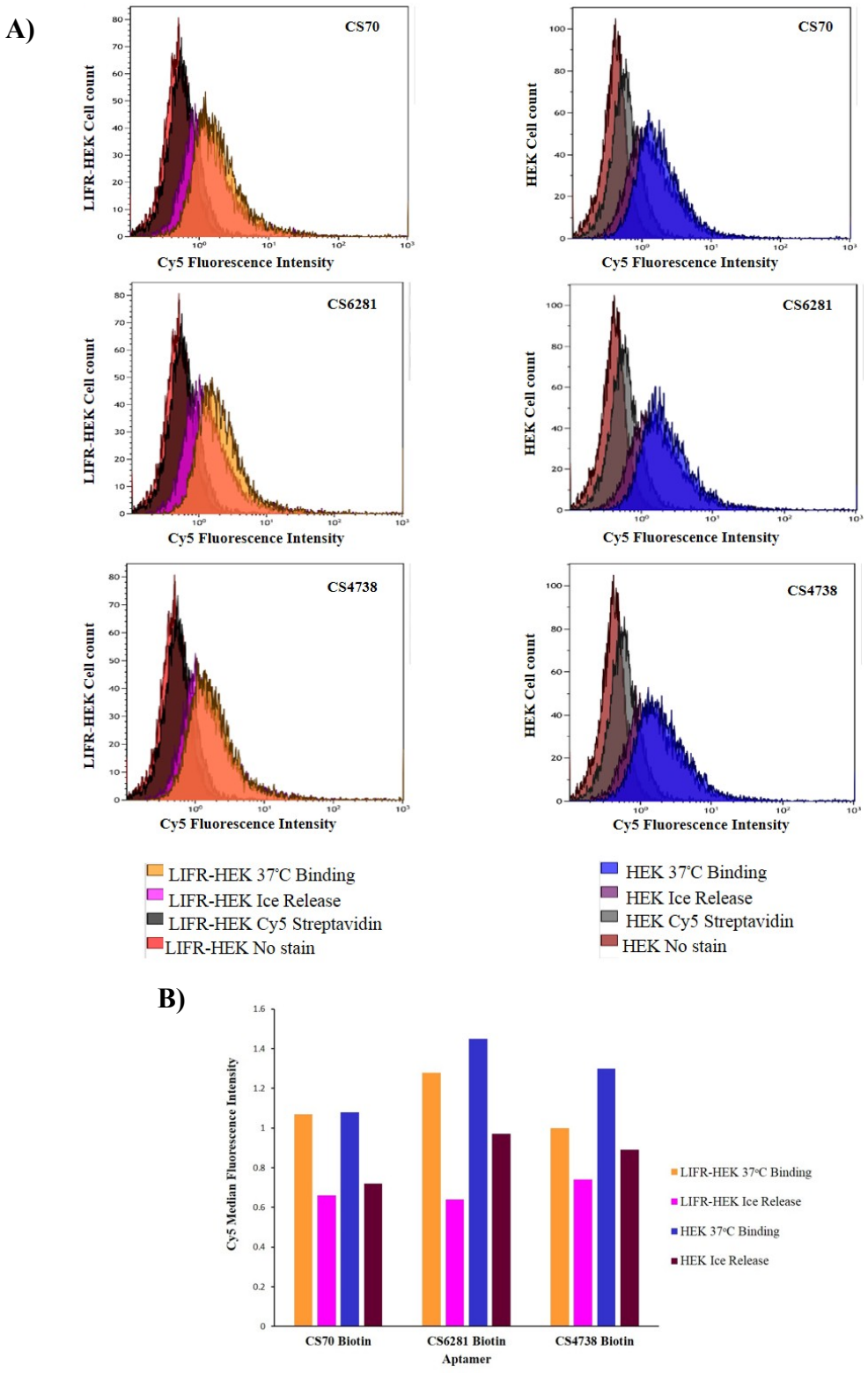


Figure 16. Cold-dependent release of three biotinylated aptamer candidates from LIFR expressing HEK and HEK cell lines. A) Flow cytometry release assays were performed with LIFR expressing HEK (LIFR-HEK) or HEK cell samples containing $1x10^6$ cells/mL incubated with 500 nM of biotinylated cold switchable aptamer candidate pre-stained with 10 µg/mL of Cy5 conjugated streptavidin (Cy5-Strep).

Negative controls for flow cytometry were prepared with $1x10^6$ cells/mL and 10 µg/mL of Cy5-Strep without aptamer or $1x10^6$ cells/mL only to control for non-selective Cy5-Strep binding and autoflorescence, respectively. Following incubation of all samples for 60 min at 37°C in the dark, an aliquot of each cold switchable biotinylated aptamer candidate binding sample was incubated on ice for 30 min and was termed the ice release sample. Cy5 fluorescence intensity distributions presented are representative of the live cell population for each sample. B) Comparison of the Cy5 median fluorescence intensity of LIFR-HEK and HEK cell samples, previously stained with cold switchable biotinylated aptamer candidates at 37°C, before and after incubation on ice. Cy5 median fluorescence intensity of each cell sample was corrected for non-selective Cy5 Strep staining by subtracting the Cy5 median fluorescence intensity for the corresponding Cy5-Strep alone control from each cell line.

3.6 Summary of cold switchable aptamer candidate release activity

The ability of each cold switchable (CSW) aptamer candidate to release from LIFR-HEK or HEK cells was quantified using the percent reduction in Cy5 median fluorescence intensity of CSW aptamer candidate bound cell samples following incubation on ice. This was calculated from the difference in median Cy5 fluorescence intensity between the CSW aptamer 37°C binding sample not incubated on ice and an aliquot of this sample incubated on ice for 60 min (CSW ice release sample). This difference was expressed as a percentage of the Cy5 median fluorescence of the CSW aptamer binding sample not incubated on ice. The calculated percent release refers to the percentage of CSW aptamer candidates originally bound to LIFR-HEK or HEK cells at 37°C which were released from these cells following cooling of the sample on ice.

The percent release of CS70, CS6281 and CS4738 biotinylated CSW aptamer candidates from LIFR-HEK cells following incubation on ice was 38%, 50% and 26%, respectively, while the percent release of these biotinylated CSW aptamer candidates from HEK cells after incubation on ice was 33%, 33% and 32%, respectively (Figure 17). Therefore, all three biotinylated aptamers released non-selectively from both the LIFR-HEK and HEK cell lines, irrespective of whether or not the LIFR target was expressed. Percent release for CS70 Cy5 conjugated aptamer was similar to that observed for its biotin conjugate, with 35% and 34% release from LIFR-HEK and HEK cells, respectively, after

incubation on ice (Figure 17). The LIF5 aptamer, a selective aptamer for LIFR expression, exhibited 64% release from LIFR-HEK cells (Figure 17).

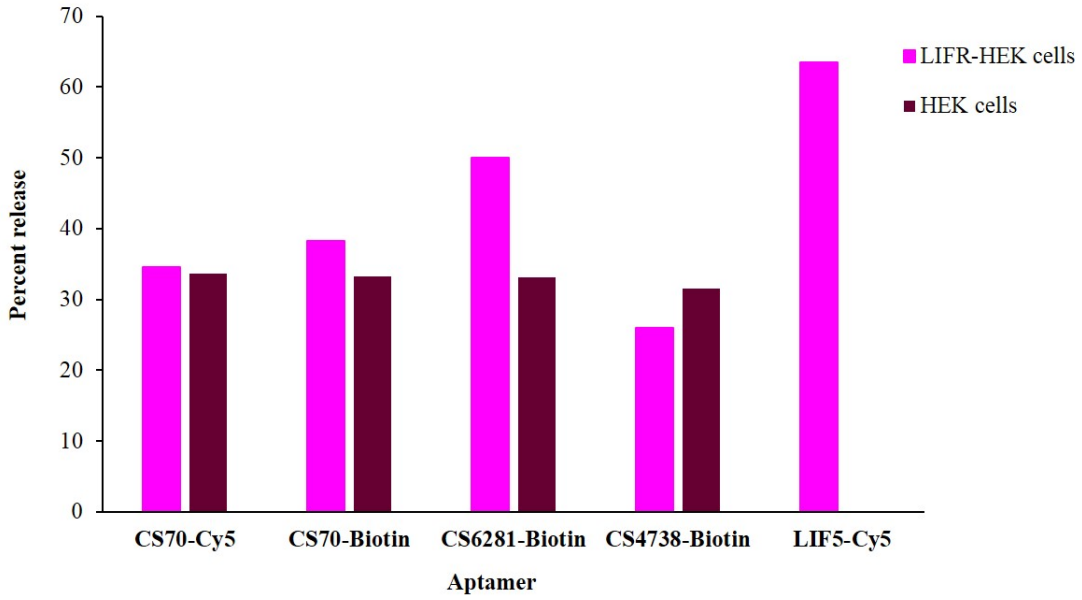


Figure 17. Percent release of cold switchable aptamer candidates from LIFR expressing HEK and HEK cells following incubation on ice. LIFR expressing HEK (LIFR-HEK) and HEK cell samples were incubated with the cold switchable aptamer candidates for 60 min at 37°C. The LIF5 aptamer, a selective aptamer for LIFR expressing cells developed by traditional cell-SELEX, was incubated for 60 min at room temperature. An aliquot of the resulting 37°C binding sample was incubated on ice for 60 min and was termed the ice release sample. Aptamer binding to both cell lines was determined by flow cytometry detection of Cy5 fluorescence emission, with biotinylated aptamers being detected with Cy5 streptavidin. Percent release was calculated as the percent decrease in CSW aptamer candidate stained cell Cy5 median fluorescence intensity (Cy5 MFI) after incubation on ice as follows; percent release = ((Cy5 MFI 37°C binding sample – Cy5 MFI ice release sample)/Cy5 MFI 37°C binding sample) x 100.

4. DISCUSSION

Immunomagnetic cell separation is an efficient technology allowing for large scale purification of target cells based on membrane biomarker expression (24). However, current removal of target cells from antibody-magnetic bead conjugates relies on the addition of exogenous agents to denature the antibodies or compete for target binding which can alter target cells during the elution stage of the procedure (29). An alternative proposal investigated in this research project, was to develop aptamers which switch conformation when incubation temperature is decreased from 37°C to 4°C, allowing the release of target cells during the elution stage of magnetic bead cell separation. Previous studies have established that riboswitches, the *in vivo* equivalent of an RNA aptamer, rely on both Mg^{2+} concentration and temperature to signal structural conformations altering target binding (66). The *in vitro* evolution of aptamers by cell SELEX was therefore modified to evolve aptamers which bound to LIFR expressing target cells at 37°C and release these cells following incubation on ice, an elution protocol anticipated to have limited impact on cell viability. Following six rounds of this novel cold switchable (CSW) SELEX protocol (Figure 5), three aptamer candidates were screened by flow cytometry and exhibited affinity for LIFR expressing HEK cells at 37°C and evidence of cooling dependent release from these target cells. However, these aptamer candidates were found not to be selective binders for the target receptor, LIFR.

The positive selection phase of the CSW SELEX protocol was successful, as the sixth round CSW SELEX (CSW6) pool exhibited 3-fold greater binding to LIFR expressing HEK cells at 37°C than did the randomly generated sequences of the DNA library (Figure 9A). CSW SELEX started with a twelfth round cell SELEX pool, originally selected from the DNA library, which was enriched for LIFR selective binders at physiological temperature. Therefore, it was expected that the CSW SELEX pool would exhibit enhanced binding to LIFR expressing HEK cells at 37°C. Confirming this result, an aptamer candidate selected from the CSW6 pool, CS70-Cy5, exhibited significantly greater binding to LIFR expressing HEK cells at 37°C than the DNA library (Figure 12B). This provides evidence of selective pressure being applied during CSW SELEX to enrich for DNA molecules which have affinity for LIFR

expressing HEK cells. In a pilot experiment, biotinylated CS70, CS6281 and CS4738 aptamer candidates caused a 2.8-fold, 3.2-fold and 2.7-fold shift, respectively, in LIFR expressing HEK cell median Cy5 fluorescence intensity compared to LIFR-HEK cells incubated with Cy5 streptavidin detection agent alone (Figure 16A). To determine if the binding of the biotinylated aptamer candidates to target LIFR expressing HEK cells is significant, the assay will need to be repeated at least three times and compared to a biotinylated library control. Based on the initial results from the biotinylated flow cytometry aptamer binding assay, it appears that CS6281 and CS4738 aptamer candidates have affinity for LIFR expressing HEK cells similar to that of CS70. Therefore there is evidence that CS6281 and CS4738 also have been selected from the DNA library due to their enhanced affinity for LIFR expressing HEK cells at 37°C.

In further studies, it was demonstrated that the binding of the three aptamer candidates to recombinant LIFR expressing HEK cells was not selective for the target LIFR as the aptamers exhibited the same degree of binding to HEK cells. This conclusion was based on flow cytometry binding assays in which LIFR expressing HEK cells incubated with CS70-Cy5 at 37°C exhibited no measurable difference in Cy5 median fluorescence intensity compared to that of untransduced HEK cells incubated with CS70-Cy5 (Figure 12B). As both LIFR expressing HEK and HEK cells incubated with CS70-Cy5 at 37°C had significantly greater Cy5 median fluorescence intensity compared to the respective cell line incubated with DNA library, it is likely that CS70-Cy5 binds to another component endogenously expressed on the HEK cell membrane. A similar trend was observed with the CS70, CS6281 and CS4738 biotinylated aptamer candidates in which HEK cell Cy5 median fluorescence intensity, as detected by Cy5 streptavidin, was equal to or greater than that of LIFR expressing HEK cells following incubation with the CSW aptamer candidates at 37°C (Figure 15B). Therefore, the negative selection step in the CSW SELEX procedure was not effective in eliminating aptamers which bound to HEK cells at 37°C. Improvement of the negative selection procedure is necessary to develop CSW aptamers that are selective for a cell surface biomarker of interest for purification of target cells in a magnetic bead based isolation procedure. To accomplish this the number of CSW SELEX rounds containing a negative selection phase

should be increased to five or six rounds. In addition, the duration of DNA molecule incubation with HEK cells during negative selection should be lengthened from 30 to 60 min in order to provide stringent elimination of non-selective binders.

The selection of non-selective aptamer candidates from the CSW SELEX protocol was not anticipated primarily because the original cell SELEX pool used for selection was already enriched for DNA molecules with affinity for LIFR at 37°C. The goal of the CSW SELEX protocol was to select DNA molecules from this pool which release LIFR expressing cells after incubation on ice. However, it appears that during the CSW SELEX protocol non-selective binding of DNA molecules has been enriched for in the pool of potential LIFR aptamer candidates. It could be postulated that due to the recovery of DNA molecules which release the target cell line following incubation on ice, weak binders from the original LIFR cell-SELEX pool have been amplified during selection. However, this is in contradiction to the flow cytometry screening results of CSW aptamer candidates which have on average 3-fold greater binding to LIFR-HEK cells than LIF6 biotinylated aptamer (Figure 15B). In addition the CS70-Cy5 aptamer exhibited significant 7-fold greater binding than LIF5-Cy5 labeled aptamer to LIFR expressing HEK cells (Figure 12C). LIF5-Cy5 and LIF6-biotin are positive controls developed by cell SELEX which exhibit selective binding for LIFR expressing HEK cells.

The flow cytometry binding analysis of the CSW6 pool, from which the non-selective aptamer candidates were chosen, showed that DNA molecules within this pool had a 2-fold greater binding to LIFR expressing HEK cells compared to HEK cells (Figure 9A). This is not an optimal result as there is a 50% possibility of choosing a candidate sequence which is not selective for LIFR and more stringent negative selection procedures should be used in the future. However, it is possible that LIFR selective aptamer candidate sequences remain as yet unscreened from the CSW SELEX sixth round pool. Therefore, performing a more conservative sequencing analysis of this CSW SELEX pool could result in the removal non-selective binders to LIFR prior to ordering aptamer candidates. In the present sequencing analysis non-selective binders are controlled for by performing a phylogenetic tree analysis of multiple

aptamer pools evolved by cell SELEX to bind different receptors using the same HEK *in vitro* expression system. Therefore, clusters in a phylogenetic tree which contain DNA sequences from different cell SELEX pools most likely bind to a common component of the HEK cell membrane and not the target receptor of interest. However, this analysis can be liberal and some S1P receptor pool DNA molecules are present in a cluster of the phylogenetic tree dominated by LIFR CSW aptamer candidate sequences (Figure 10B). An alternative analysis can be performed by Galaxy bioinformatics software in which the entire pool of unique sequences recovered from CSW SELEX can be compared with all sequences from other pools and common sequences removed (Compare two datasets tool, (65)). The disadvantage to this technique is that it can be too conservative as CSW SELEX pool sequences must be identified by a barcode forward primer sequence and any PCR mutations within this sequence will force the software to consider the DNA molecule from another pool, thereby removing potentially selective aptamer candidates.

The pool of DNA molecules recovered from six rounds of CSW SELEX and the three aptamer candidate sequences selected from this pool showed evidence of cooling dependent release from both LIFR expressing HEK and HEK cell lines. Flow cytometry analysis of cold dependent release involved incubation of a cell sample with Cy5 labeled SELEX pool or aptamer candidate at 37°C and then one half of this solution was immediately analyzed by flow cytometry, while the second half of the solution was analyzed following incubation on ice. The percent reduction in Cy5 median fluorescence intensity of the cell sample due to incubation on ice reflected the percent release of fluorescent DNA molecules which had originally bound LIFR expressing HEK or HEK cells at 37°C. The sixth round CSW SELEX pool exhibited 85% release from LIFR expressing HEK cells after incubation on ice for 30 min (Figure 9A). All CSW aptamer candidates required 60 min incubation on ice to yield an appreciable release from LIFR expressing HEK or HEK cell lines (Figure 16A). The most promising aptamer candidate in terms of cold switching activity was CS6281-biotin which exhibited 50% release from target LIFR expressing HEK cells, as well as 33 % release from control HEK cells (Figure 17). The other aptamer candidates CS70-

Cy5, CS70-biotin and CS4738-biotin exhibited 35 %, 38 % and 26% release from LIFR expressing HEK cells, respectively, as well as 34 %, 33 % and 32 % release from HEK cells, respectively (Figure 17). CS70-Cy5 and CS70-biotin aptamers had similar percent release for cold switching activity even though the sensitivity of the later detection method for aptamer candidate binding was lower. (Figure 17). In future studies these release assays need to be performed at least three times to determine if there is a significant reduction in Cy5 median fluorescence intensity of aptamer candidate stained cells due to change in temperature from 37°C to 4°C. Furthermore, while these aptamer candidates provide evidence for cooling dependent release, the optimum result would be 100% release of LIFR expressing HEK cells due to incubation on ice. This would imply that all aptamers which had originally bound the target cells at 37°C have been released. The need to develop aptamers that exhibit close to 100% release, as determined by a significant shift of target cells to the level of cell autofluorescence, is to ensure high recovery yields for magnetic bead based cell separation using the proposed elution on ice protocol. One way to improve cold dependent release would be to gradually reduce the incubation time on ice during rounds of CSW SELEX. This would apply more selective pressure to recover DNA molecules that undergo substantial conformational changes due to incubation on ice and therefore have enhanced target cell release activity.

In this study it was shown that LIF5 aptamer, which was previously developed by cell SELEX to bind LIFR expressing cells selectively, had 64% release from LIFR expressing HEK cells due to 60 min incubation on ice (Figure 14). Considering that cooling dependent release was not one of the selection criteria during evolution of this LIFR selective aptamer, it is interesting that this aptamer should release from LIFR-HEK cells at 4°C. This aptamer even showed a shift in Cy5 fluorescence and therefore aptamer binding after 30 min on ice under which conditions CSW aptamer candidates showed negligible release (data not shown). Both CSW aptamer candidates and LIF5 are derived from the same twelfth round LIFR SELEX pool, however, LIF5 sequence was not recovered in the sixth round CSW SELEX pool. In addition, LIF5 shows less than 50% sequence similarity with the three CSW aptamer candidates. This assay will need to be repeated at least three time to determine if this result is significant. In addition,

it would be of value to test more LIFR selective aptamers developed in our lab for ice dependent release, to determine whether LIF5 aptamer was a rare find. If more LIFR aptamers, which were not selected using CSW SELEX, are found to release LIFR target cells on ice it would indicate that ice incubation is not only causing a change in aptamer conformation, but is possibly resulting in additional changes of the HEK cell membrane environment promoting release. As LIF5 is selective for LIFR, unlike the CSW aptamer candidates, future studies could be performed using LIF5 aptamer in a streptavidin coated magnetic bead assay to isolate LIFR expressing HEK cells. The purpose would be to test the cooling dependent elution strategy in this affinity chromatography based cell isolation assay.

An area for improvement in this study is the optimization of flow cytometry detection of biotinylated aptamer candidate binding with Cy5-labeled streptavidin. This detection system has been successfully used before in previous studies to screen cell SELEX aptamer candidates by staining target cells post incubation with biotinylated aptamer for 30 min with 10 µg/mL of Cy5 labeled streptavidin (67). However, using these conditions in the present study, Cy5 fluorescence shift of LIFR-HEK cells due to CS70 biotinylated aptamer candidate binding could not be resolved from background cell autofluorescence. This was unexpected as the Cy5 labeled form of this aptamer candidate had already been characterized by flow cytometry to exhibit significant binding to LIFR expressing HEK and HEK cells (Figure 12C). The end result of multiple optimization protocols was that Cy5 fluorescence shift of target cells due to biotinylated aptamer could be resolved from background autofluorescence only when biotinylated aptamers were incubated with the Cy5-streptavidin before addition of these aptamers to target cells (Figure 15A). However, the shift in LIFR-HEK cell Cy5 median fluorescence intensity from cell autofluorescence due to addition of CS70 aptamer candidates was 7-fold for Cy5 labeled and only 3-fold for the biotin labeled candidate, in spite of the fact that a 2-fold greater concentration of the biotinylated aptamer candidate was used in the assay (Figure 12A, Figure 15A). To improve biotinylated aptamer detection, a possibility would be to increase the ratio of Cy5 labeled streptavidin to biotinylated aptamer, as one streptavidin labeled fluorophore can theoretically bind four biotinylated aptamers thereby

reducing sensitivity. However, increasing the concentration of Cy5 streptavidin 4-fold risks increasing non-selective binding of the detection agent to target cells without aptamer present. The finding in this assay that biotinylated aptamers required incubation with Cy5-streptavidin prior to being added to target cells has implications for streptavidin coated magnetic bead cell isolation using biotinylated aptamers. Biotinylated aptamers may need to be incubated with the magnetic beads prior to adding these aptamer-bead complexes to the target cells. This procedure has been accomplished previously, however there is evidence that free affinity ligands have better ability to capture their targets compared to when the affinity ligands are complexed with a magnetic bead (24).

One aspect of this study on cold switchable aptamer development which requires further investigation is testing the ability of biotinylated CSW aptamer candidates that selectively bind a target receptor to isolate target expressing cells in a streptavidin coated magnetic bead cell separation assay. The three current candidates are not selective for LIFR, therefore prior to use in a cell separation assay the membrane receptor(s) they bind on the HEK cell membrane would need to be identified. This can be accomplished using a procedure termed aptamer facilitated biomarker discovery through the use of aptamer-receptor crosslinking, purification and mass spectroscopy identification of aptamer targets (68). However, an alternative strategy would be to modify the negative selection stage of CSW SELEX or the selection of candidate sequences from the already developed CSW SELEX sixth round pool to identify an LIFR selective CSW aptamer. The ability of this aptamer to isolate LIFR expressing HEK cells using streptavidin coated beads and elution through ice incubation can be tested using a mixture of recombinant HEK cell lines which express multiple protein targets. The percentage of LIFR positive cells before and after CSW aptamer-magnetic bead based cell separation would be identified by anti-LIFR monoclonal antibody in a flow cytometry analysis. This assay has already been performed in our lab to validate EDTA switchable aptamers for LIFR expressing HEK cells. In addition the recovery, purity and viability of cells recovered using the cooling dependent elution strategy with CSW aptamers would need to be compared with current antibody technology elution methods such as DETACHaBEAD anti-FAB antibodies (Life

Technologies). The end goal would be the isolation of LIFR positive cells from human tumors in order to identify if these isolated cells are enriched for self-renewal activity, which would confirm previous reports of LIFR upregulated expression in cancer stem like cells (69, 70).

The evidence for release seen in this study with the three CSW aptamer candidates identifies a novel property of aptamers, the ability to change conformation at lower temperature and allow viable cell release, which cannot be accomplished with antibodies. While the propensity for DNA to form more complex motifs and internal base pairing at lower temperatures provides a theoretical base for how aptamer configuration could change during incubation on ice to release the target cell membrane receptor (Figure 11), it is still unclear how this occurs. In particular riboswitches *in vivo* actually have higher affinity for their target ligand at lower temperatures (66). Therefore, CSW SELEX has enriched for a rare class of molecules. The mechanism of cooling dependent target release, whether it be by aptamer, target protein or even cell membranome conformational change, would require elucidation by circular dichroism or X-ray crystallography techniques. Finally, in order to create a robust platform for the development of cold switchable DNA aptamers for cell isolation based on biomarker expression the CSW SELEX protocol must be efficient and easy to reproduce regardless of the biomarker target. The PCR amplification of CSW SELEX pool DNA molecules required multiple rounds of optimization which were not consistent between CSW SELEX rounds. Therefore, further optimization of CSW SELEX procedure will be required to achieve a consistent CSW aptamer development platform comparable to established antibody elution strategies (71).

In summary, this study has examined the selection and properties of aptamers developed by a modified cell SELEX procedure to bind LIFR expressing cells at 37°C and release these target cells following incubation on ice. The cold switchable aptamer candidates screened in this study exhibited binding to both LIFR expressing recombinant HEK cells and HEK control cells at 37°C. They subsequently demonstrated as great as 50% release of LIFR-HEK cells or 33% release of HEK cells after incubation on ice. The modified CSW SELEX protocol can be used to develop cold switchable aptamers

for any cell surface marker of interest for cell sorting using an *in vitro* HEK cell line for target expression. In order to validate the use of these cold switchable aptamers for facile elution of viable cells in magnetic bead based affinity chromatography, both the negative selection and release on ice stages of CSW SELEX will need to be further optimized to improve selectivity and enhance the cold-dependent switching ability of evolved aptamers.

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6. APPENDIX

R script and console output for Type 1 ANOVA analysis of CS70-Cy5 CSW aptamer candidate flow cytometry binding assay data.

Rectangular Data Matrix

Aptamer	Cell Type	MFI
CS70	LIFR	3.02
CS70	LIFR	3.49
CS70	LIFR	2.68
CS70	HEK	3.21
CS70	HEK	2.65
CS70	HEK	3.9
LIF5	LIFR	1.64
LIF5	LIFR	1.4
LIF5	LIFR	1.25
LIF5	HEK	0.52
LIF5	HEK	0.57
LIF5	HEK	0.44
Library	LIFR	0.26
Library	LIFR	-0.02
Library	LIFR	0.39
Library	HEK	0.18
Library	HEK	-0.03
Library	HEK	0.14

```
anova.model1 <- lm(MFI ~ Aptamer + Cell.Type + Aptamer:Cell.Type, contrasts = list(Aptamer = contr.sum, Cell.Type = contr.sum),     data = aptamer)

> shapiro.test(residuals(anova.model1))

        Shapiro-Wilk normality test

data:  residuals(anova.model1)

W = 0.957, p-value = 0.5452

> leveneTest(MFI ~ Aptamer * Cell.Type, data = aptamer)

Levene's Test for Homogeneity of Variance (center = median)

          Df      F value         Pr(>F)

group   5         1.5061          0.2593

          12
```

Type 1 ANOVA Analysis of Variance Table

Response: MFI

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Aptamer	2	28.9715	14.4858	132.3304	6.659e-09 ***
Cell.Type	1	0.3556	0.3556	3.2485	0.09664 .
Aptamer: Cell.Type	2	0.9874	0.4937	4.5101	0.03462 *
Residuals	12	1.3136	0.1095		

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Pairwise comparisons using t tests with pooled SD

data: MFI and Aptamer.Cell.Type

	CS70 HEK	CS70 LIFR	Library HEK	Library LIFR	LIF5 HEK
CS70 LIFR	0.99054	-	-	-	-
Library HEK	9.7e-07	1.7e-06	-	-	-
Library LIFR	1.4e-06	2.4e-06	0.99054	-	-
LIF5 HEK	3.3e-06	6.6e-06	0.60772	0.86560	-
LIF5 LIFR	0.00018	0.00046	0.00241	0.00424	0.02608

P value adjustment method: holm