Aptamer Selection for Cancer Stem Cell Marker CD44

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1-Abstract:

Cancer is a difficult disease to characterize because of the variation with in its subtypes. Cancer stem cells (CSCs) are a subset of cancer cells that can self-renew and differentiate into any type of cancer. Adhesion receptor, CD44, which is the primary receptor for hylauronic acid, has been associated with cancer stem cells and tumour growth, as well, is known to be the most common surface marker to identify CSCs. Furthermore, CSCs are determined to be responsible for cancer tumour formation, chemo-therapy resistance and recurrence. There is a need for a molecular probe that acts on identifying CD44 receptor on these cells, allowing for their isolation and study. Aptamers are single stranded oligonucleotides selected from a random sequence pool. They tend to exhibit high affinity and specificity for their targets, and hence, can act as probes. In this study, the systematic evolution of ligands by exponential enrichment (SELEX) is used to isolate ssDNA aptamers that can bind CD44 receptor with high affinity. A Lentiviral vector was used to transfect 293T cell line with CD44 gene, allowing for creation of two cell lines, one expressing the gene of interest, while the other lacking endogenous expression. Using these cell lines, it was possible to select aptamers of high affinity to the target on whole-cell environments. The enrichment of selected aptamers pools was monitored by flow cytometry binding assays. Generation of ssDNA aptamer pools were done using two different methods: linear after exponential PCR (LATE PCR) and lambda exonuclease digestion, in order to optimize the selection process. Aptamers can act as a method to exploit CD44-hylauronic acid interactions for targeted drug delivery for cancer stem cells as well as inhibiting this interaction leading to inhibition of metastasis, invasion and cancer recurrence. From this project, pools 5 and 6 generated using LATE PCR showed preferential binding towards CD44+ 293T cells, which suggests possible aptamers for CD44 receptor.

Acknowledgments

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Statement of contribution

Experimental Concept

The idea of modified cell-SELEX was conceptualized by Shahrokh Ghoboloo, and the basic idea was adapted from Darija Muharemagic protocol. Some modifications were conceptualized by Noreen Ahmed. Method 2 of cell-SELEX used in this project (involving lambda exonuclease digestion) was conceptualized by Shahrokh Ghoboloo.

The lentivirus transfection to produce CD44+ 293T cell line was conducted by Shahrokh Ghoboloo.

Experimental Testing

All rounds of Selection were performed by Noreen Ahmed. Training for the flow cytometry was done by Shahrokh Ghoboloo and Nadia Al-Youssef. All affinity testing and analysis was done by Noreen Ahmed and Shahrokh Ghoboloo.

The thesis was written and edited by Noreen Ahmed

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2-Introduction

In the recent years, the role of stem cells has been reviewed for their ability to give rise to different types of tissue. Their ability to self renew and differentiate is of interest to many researchers. With greater understanding of their biology and properties, their role in malignancy and tumour formation has been addressed. One of the major issues in cancer is the variation within the disease itself. There are multiple different subtypes that make it harder for specific targeting of the disease. Most therapies available for cancer are not specific and therefore lead to relapse and recurrence (Reya T. et al. 2001). Cancer stem cells (CSCs) are proposed to be a population of cancer that are present in small percentages in all cancer subtypes (J. E. Dick, 2003). They have the ability to self-renew and differentiate in to many types of cancer, the same way normal stem cells do in order to maintain different types of tissues (Reya T. et al. 2001). Identifying specific markers for Cancer stem cells has been of great interest as a potential detection method for these cell populations. Identification of CSCs has a large therapeutic potential. One of the most important Bio-markers is CD44, a cell adhesion receptor. The purpose of this research is to select aptamers to putative cancer stem marker CD44. This marker has been implicated to play an important role in providing cancer stem cells with their invasive tumourgenetic properties (Ricardo et al. 2011). Aptamers have the potential to act as probes that can help in characterization and identification of cancer stem cells in a cancerous tumour population.

2.1 Cancer Stem Cells and CD44 Biomarker

Cancer is a broad term that encompasses many diseases. Despite the advancement in diagnosis and identification methods, it is still one of the most leading causes of death in the world

(Globocan, 2008). However, in general, it is simply identified as a compilation of mutations inside a cell leading to uncontrollable cell growth. The maintenance and repair of many adult tissues are controlled by a population of cells named stem cells. They are characterized by the ability to self renew and differentiate to different type of cells in order to help maintain different types of tissues (Guo et al, 2006). Most recent evidence has suggested that similar subpopulations of cells are present in cancer. This subpopulation is known as cancer stem cells (CSCs) (Guo et al., 2006). As the name suggests, they hold similar properties as healthy stem cells. They act the same way as adult stem cells, where they are able to self renew and give rise to all other cell tumour types (Dean M, 2006). Furthermore, there have been an abundant amount of evidence that associate cancer stem cells with the tumourigenecity and invasiveness of cancer (visvidar et al. 2008). These populations of cells have also been directly related with metastatic ability of most tumour cells (Visvidar et al. 2008). Cancer stem cells constitute a very small percentage of cells in every tumour mass. They are typically 0.1% to a few percent of any cancerous tumour (Gao JX, 2008). Typical therapies available, such as chemotherapies and other established strategies destroy most tumour cells; however, they do not eliminate the source— cancer stem cells. These properties have been directly related to setting the stage for tumour relapse (Guo et al. 2006). It has been reported that therapies targeting cancer stem cells will cause survivals in patients, specifically those with a metastatic disease (Reya T. et al. 2001). Furthermore, the ability to isolate cancer stem cells for each cancer type, will allow the exploration of mutations and disruptions that cause these cells to act irregularly and therefore, this can be used as the basis of understanding for developing specific drugs and therapies that could help destroy cancer cells and prevent their relapse (Reya T. et al. 2001).

Cancer stem cells cannot be targeted by conventional methods such as chemotherapy and radiation therapy, which is because those therapies tend to target highly proliferative cells (a characteristic of normal cancer cells), however cancer stem cells have been identified to spend most if their life-time in G0 phase, which a slow rate growth/proliferative stage (Reya et al., 2008). Therefore, conventional cancer treatments cannot be used to target CSCs, causing relapse and recurrence of cancer.Eradication of this cancer population is the new insight for cancer treatments. The major challenge to be faced, however, is the characterization and isolation of this subpopulation (AlHajj et al. 2003). Many recent studies have been focused on identifying a biomarker that can be used to isolate these cells (AlHajj et al. 2003). One of the major markers identified is CD44, a cell adhesion hyaluronic acid receptor. It is one of the most commonly studied receptor for CSCs identification.

CD44 is a multi-structural, multifunctional class I transmembrane glycoprotein (ponta et al. 2003). It is a receptor for hyaluronic acid and it plays a role in promoting the migration of cells. CD44 has been identified to be overly expressed in many cancer types (both in its variant or standard form) (Du et al. 2003). Some of its functions are related to regulation of cell adhesion, proliferation, cell survival, cell motility, differentiation, cell growth and angiogenesis. It has also been associated with presenting receptors with their proper cytokines and chemokines (Lesley et al. 1998). In addition to hyaluronic acid, CD44 interacts with other ligands. CD44 was shown to interact with osteopontin, which was related to tumour progression (Jagupili et al. 2011). Also, it was found to interact with laminin, collogen and fibronectin (Jagupili et al. 2011). Since CD44 is an adhesion receptor, it was implicated that it plays a great role in metastasis by helping cancer cell to adhere to blood vessel walls (Jagupili et al. 2011). Furthermore, it has been related to a complex network of signalling cascades that promote and enhance tumour interactions and

initiations; these include promotions of interactions with receptors such as tyrosine kinase Her2 (Jagupili et al. 2011). Additionally, CD44 is expressed in almost all cancer stem cell types including but not limited to; Human Breast Cancer (de la Torre, Heldin, & Bergh, 1995), Endometrial Cancer (Fujita & Sato, 1995), Colon Cancer (Herrlich, Pals, & Ponta, 1995), Liver Cancer (Jonas & Allenmersh, 1995), Renal Cancer (Kan, Aki, et al., 1995), Bladder Cancer (Matsumura et al., 1995), Cervical Cancer (Kainz et al., 1996), Skin Cancer (Simon et al., 1996), Lung Cancer (Ochiai et al., 1997) and Ovarian Cancer (Stickeler, Mobus, et al., 1997).

Many different researches have correlated cancer recurrence and relapse to the levels of CD44 expression in cancer stem cells. It has been reported that an identified a humanized monoclonal antibody specific for CD44 that targets and inhibits it, directly kills chronic lymphocytic leukemia cells (Suping Zhang, et al, 2013). It has also been reported that targeting CD44 with α-CD44 antibody reduced the number of acute of acute myeloid leukemic stem cells, which provides good evidence that CD44 can act as a potential cancer stem cell marker and a target for therapy. Furthermore E Marangoni et al, have identified that targeting breast cancer stem cells by inhibiting CD44 using α-CD44 antibody reduces tumour growth and prevents post-chemotherapy relapse in human breast cancer patients. Additionally, targeting CD44 in Hepatocellular Carcinoma cell lines using SiRNA decreased growth and invasion in soft agar (Xie Z et al, 2008). Moreover, a study was conducted to further investigate the role of CD44 in ovarian cancer relapse and metastasis. It was found that treatment of nude mice xenografts models with anti-CD44 antibody lead to decrease in tumour implants significantly, leading to a solid connection between the progression of cancer and CD44 (Strobel T. et al., 1997). Thus, inhibition of cancer can be conducted through targeting CD44. Furthermore, it was shown that using microRNA, specifically targeting CD44, miR-34a leads to inhibition of

proliferation of prostate cancer stem cells (Liu, c. et al, 2011). All the previous evidence solidifies the importance of CD44 in cancer detection and inhibition, and validates it role as CSC marker.

Even though CD44 has been implicated as a cancer stem cell marker, it is not universally expressed on cancer stem cells or limited to cancer stem cells only (jagupili et al, 2011). Many studies refer to CD44 as a highly expressed surface marker in many types of cancer stem cells ranging between 80% to 85% expression level (jagupili et al, 2011). However, it is important to understand that no marker can be used to universally identify CSCs in various cancers (since CSCs tend to act differently depending on the cancer type itself). Regardless, it is certainly acceptable that CD44 is one of the best identifiable biomarkers available (Cichy J et al. 2003). Another issue that rises is the fact that CD44 is available in many isoforms and variants depending on the type of tumour (Cichy J et al. 2003). Therefore, CD44 would only act as a gateway for detection of CSC or as an isolation start point.

Consequently, there is an absolute need for a method of detection and identification that would allow for a quick way to isolate these cell populations. Aptamer selection methods, in addition as acting as a detector and diagnostic method, it will provide a way to study CD44 receptor overtime.

2.2 Aptamers

Aptamers are single stranded molecules either ssDNA or ssRNA. They possess the ability to form secondary structures and complex three dimensional conformations. They have the ability to form many different types of shapes including loops and helices. Their potential to change their conformation gives rise to their affinity of binding to a wide range of targets with great

specificity and high sensitivity (Song et al. 2012). Research groups all over the world have selected aptamers for different targets including proteins, small molecules, whole cells, cell surface receptors and viruses. Furthermore, aptamers have been used in many areas in research and industry (Eugene et al., 2006). They have been used in areas such as pharmaceuticals, analytical chemistry and other environmental applications. A very distinctive and successful aptamer that is used today is pegaptanib, which is now marketed by Pfizer, targets VEGF receptor as a treatment of wet macular degradation (Eugene et al. 2006). Other aptamers are also being selected for different targets including bloodstream targets, or cell surface targets (Sefah et al. 2012).

Aptamers were found to have high therapeutic potential that allows it to be a competitive candidate for further research and investigations. They are frequently being compared to antibodies and small molecules, due to their characteristics. Like antibodies, they share many properties. Potentially aptamers can act as available replacement to antibodies due to the wide range advantages they hold over antibodies (Sefah et al. 2012). When compared to antibodies, aptamers are produced in larger quantity in a laboratory setting. They are cheaper to produce and can be modified easily by simple chemical reactions and well defined methods in the lab (Bunka et al. 2006). The production of Aptamers does not depend of bacteria, cell cultures or animal models; instead, the process of aptamer production is highly reproducible (Sefah et al. 2012). Another advantage that is added to aptamers is they lack hydrophobic cores that the proteins (antibodies) have, and therefore they do not aggregate. Even though both aptamers and antibodies denature at higher temperatures, Aptamers tend to regain their original secondary conformation once the temperature is lowered (Sefah et al. 2012). In contrast, antibodies are irreversibly denatured, thus they do not regain their structure and this leads to loss of function.

Furthermore, low toxicity and immunogenicity are of an advantageous characteristic to aptamers, and thus they do not mount an immune response in vivo (Song et al. 2012). Moreover, they are easily modified which yield an advantage in terms of drug delivery. For example, biochemical modifications to aptamers need to be performed in order to increase the half-life of the aptamers, increase nuclease resistance, and prevents from renal infiltration (Sefah et al. 2012). Consequently modified bases are used for this purpose, as well, conjugation of aptamers to high molecular weight molecules, such as polyethylene glycol, would alter the pharmacokinetic properties.

Selection of aptamers to specific targets can be done through a developed method termed *Systematic Evolution of Ligands by Exponential Enrichment* (SELEX) initially developed in 1990 by Szostak and gold laboratories (Ellington et al. 1990, Tuerk et al. 1990). SELEX is a method that utilizes a library of $\sim 10^{15}$ to 10^{17} aptamer sequences of the same length, typically between 50 to 100 nucleotides. Aptamers in the library consists of random sequences of a fixed length flanked with primer annealing regions of known sequences; this will be used for primer amplification by polymerase chain reaction (PCR). Also, these constant sequences on either side of the aptamers can be used for chemical modifications later on.

The selection method that is being utilized in this research will constitute the basis of SELEX. Primarily, the random aptamer library will be incubated with the target cells expressing the receptor of interest, CD44. The bound aptamers are then eluted and concentrated through PCR amplification reaction. The collected aptamer pools will then be incubated with cells that are identical to the initial cells with the receptor, except they lack endogenous expression of CD44. The selection process is performed for 10 rounds, where the binding affinity of the aptamers to the receptor of interest is tested through flow cytometry binding assays. Generation of the

ssDNA aptamers was done using two different methods; lambda exonuclease digestion and linear after exponential PCR (LATE-PCR). By using these two methods, we can identify which method is more optimal for generation of ssDNA aptamers and also allow for a more efficient aptamer selection process.

The selected aptamers to the receptor of interest can be further tested for their biological response using different *in vivo* assays. Aptamers, like antibodies, can act on the receptor by inhibiting its function, inducing cell death or apoptosis.
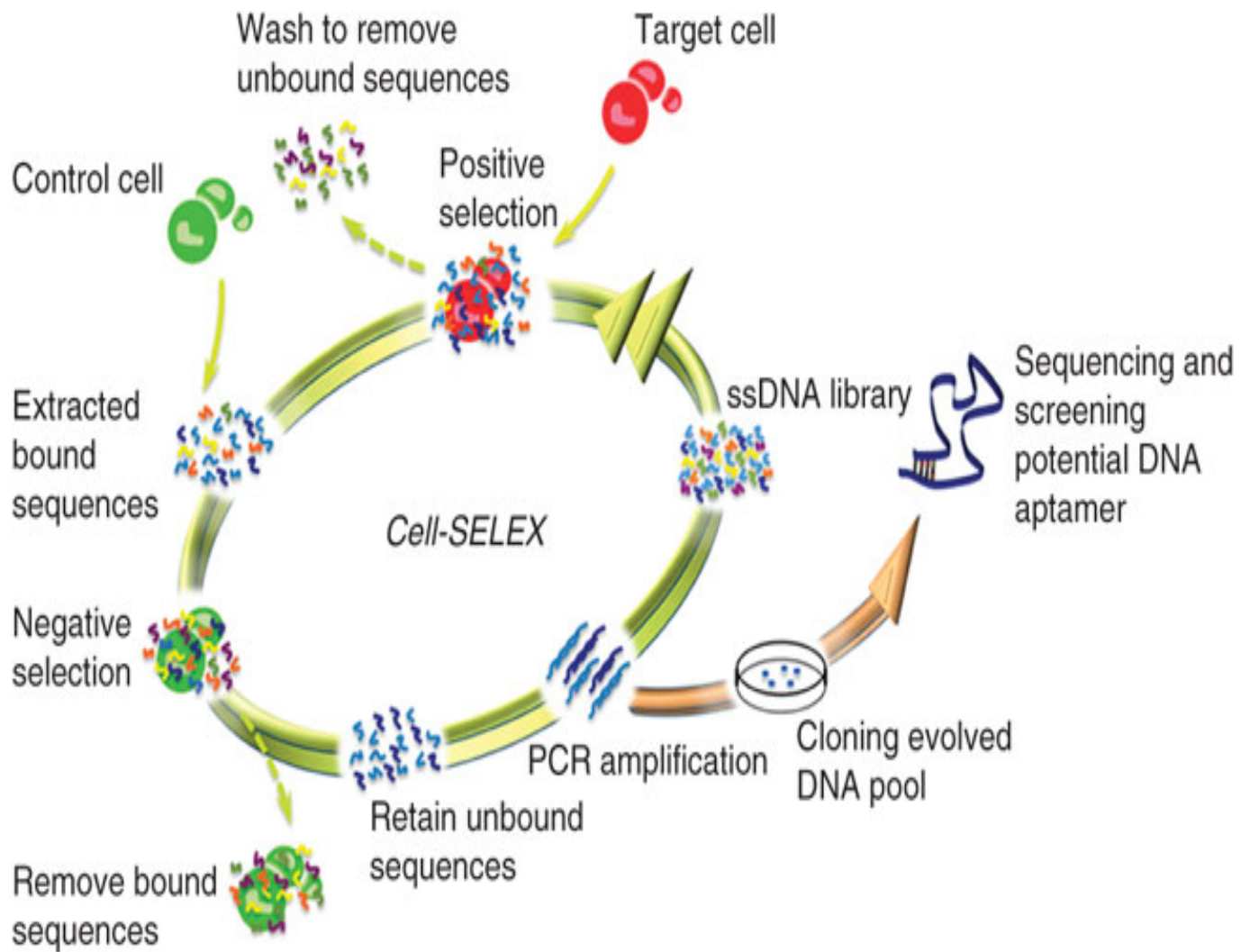
Figure 1: Proposed Cell SELEX method for aptamer selection (Sefah et al. 2010). Schematic representation of Cell-SELEX strategy for obtaining aptamers that can bind receptors in target cells. Target cells contain the receptor of interest, where positive selection would be performed. The bound sequences are then extracted and incubated with control cells that are identical to the initial cells except they lack the expression of the receptor of interest. Unbound sequences are then retained and amplified. This process this performed for 10-12 rounds, and then the obtained pools are tested for their affinity and finally the binding pools are cloned and sequenced.

Materials and Methods

3.1 Materials

Cell lines, Antibodies and Aptamers

Cell lines utilized: 293T cell line, Lenti-viral transfected CD44+ 293T cell line prepared by **Shahrokh Ghaboloo** in the Dr. Berezovski Lab. **Antibodies**: Allophycocyanin (APC) labelled mouse anti-human monoclonal CD44 antibody (BD Pharmigen-catalogue number 559942). **Aptamer library**: Harvard Library (Integrated DNA technologies). Aptamer library contains 10^{15} sequences of 100 bases in length. Aptamer used are ssDNA sequences: 60 random bases flanked by 20 nucleotides of fixed sequences for primer annealing.

Chemical Reagents

The cells used were maintained in DMEM Dulbecco's Modified Eagle Meduim (Thermo-Scientific) with 10% fetal bovine serum (Thermo-Scientific). Trypsin and phosphate buffered saline with cations and without cations (Thermo-Scientifc). Doxcycline (Sigma-Aldrich).Lenti-Viral transfection kit-Lenti-X Tet-On 3G inducible expression System kit (Clontech inc.).

3.2 Lenti-viral transfection of T293 cells (conducted by Shahrokh Ghoboloo)

Transfection of 293T cell line was performed using a prepared plasmid pLVX-TRE3G-CD44—containing the gene of interest (CD44 full length) which was cloned by Shahrokh Ghoboloo in the Berezovski Lab. The lentivirus transfection of the 293T cell line with the pLVX-TRE3G-CD44 was co-transfected with a regulatory plasmid, pLVX-Tet3G containing a regulatory gene that would allow for further control and inducible expression by expressing a trans-activator molecule upon the addition of doxycycline. The transfection process was done using LentiViral transfection kit, Lenti-X Tet-On 3G inducible expression System kit (Clontech inc.)

3.3 Flow Cytometry Verification of CD44 Expression

Lentiviral transfected CD44+ 293T cells were maintained in Dulbecco`s Modified Eagle Media supplemented with 10% Fetal Bovine Serum. The prepared media was supplemented with 1000 ng/ml doxcycline. The doxcycline induction was done for 72 hours to allow the maximal expression of the receptor on the cell surface. Once cells were 80%-100% confluent, they were ready for analysis. CD44- 293T cells were also maintained in Dulbecco`s Modified Eagle Media supplemented with 10% Fetal Bovine Serum, and will be used as a control for CD44 expression. Cells were maintained in 100mmx 20mm culture plates.Once cells were ready (both the CD44+ and CD44- cells), plates were washed with cation-free phosphate buffered saline, and cells were scrapped off using a cell scrapper. Cells were collected in falcon tubes containing 5 ml cation-free phosphate buffered saline, and the cell pellets were collected by centrifugation at 180xg for 5 minutes using Sigma 3-16 Refrigerated centrifuge (ATR,inc.).

 The supernatant containing the buffer was aspirated and the cells (both CD44+ and CD44- pellets) were re-suspended in cation containing PBS and then counted using Millipore MUSE^{TM}

Cell analyzer (EMD Millipore). Each flow cytometry sample prepared contained 100,000 viable cells. Cells were incubated with 1/50 dilution (5ng/ul) of Allophycocyanin (APC) labelled mouse anti-human monoclonal CD44 antibody for 1 hour (doxycycline inducted CD44+ cells and the CD44- cells). The antibody dilution was prepared in cation-containing PBS. Cells were then washed with cation-containing PBS and centrifuged at 180xg for 5 minutes using Sigma 3-16 Refrigerated centrifuge (ATR,inc.). Samples were then re-suspended PBS-containing buffer. Therefore, the samples available at the end were unstained CD44- , unstained CD44+, antibody stained CD44- and antibody stained CD44+. The antibody binding was analyzed by Guava EasyCyte flow cytometer.

3.4Aptamer selection:

3.4 a Positive selection

Selection round 1-10 (no negative selection performed for the first three rounds of selection)

CD44+ 293T transfected cells were grown in Dulbecco modified eagle eye media supplemented by 10% fetal bovine serum. Addition of 1000ng/ml doxycycline for induction of CD44 expression was added to the prepared media. Induction of the cells with doxycycline supplemented media was done for 72 hours, allowing the cells to become 80% to 100% confluent before the initiation of the selection process. The cells were grown and maintained in 100-mm x 20-mm cell culture dish. The aptamer selection was also performed in the 100-mm x 20-mm culture dish.

Initially, 200nM Harvard library was prepared in cation containing PBS. The prepared library was then heated at 95°C for 10 minutes then snapped cooled on ice for 5 minutes. The CD44+

293T cells were washed twice with Cation free PBS twice, and then the buffer was aspirated. Once, the cells were washed, the prepared Harvard library pool (200nM) was added to the cells and incubated at 37°C at 200 r.p.m on a incubating/cooling orbital shaker (VWR symphony^{TM}) for one hour. The total volume of library used for the selection process is 1000 ul of 200 nM library. Following the incubation, the supernatant was removed from the cells, and the cells were washed three times with cation rich PBS. Using a cell scrapper, cells were scrapped off the plate and collected. The cells were collected in 1000ul cation rich PBS buffer. The cell suspension was then heated at 95°C for 10 minutes, the centrifuged at 13,100g for 5 minutes using a PrismR refrigerated centrifuge (Biotech Inc., Montreal). This step will allow cell debris to pellet down, while the bound aptamers would be released in the supernatant. The collected supernatant was used for negative selection next. This process was performed for 10 rounds using the collected/ concentrated pools from the previous round of selection.

3.4 b Negative selection round 4-10

The positive selection for rounds 4-10 was performed similar to round 1-3, however, negative selection is performed in those rounds, and therefore there have been some minor changes.

Following the collection of the bound aptamer pool to the CD44+ 293T cells using centrifugation at 13,100xg by a PrismR refrigerated centrifuge (Biotech Inc., Montreal), the collected supernatant (aptamer pool) containing the bound aptamers (from previous round) were incubated with CD44- 293 T cells for negative selection. The supernatant was added to 293T cells that are 80%-100% confluence, maintained in Dulbecco's modified eagle Media supplemented with 10% fetal bovine serum (Thermo-Fisher). Firstly the Media was aspirated from the negative cells, and then the aptamer pool was incubated with the cells for one hour at

37°C on a slow shaker (200 r.p.m). The negative selection was also performed directly in culture dish (100 mm x 20 mm). Following the incubation, the supernatant was collected off the cells and centrifuged at 1000xg for 5 minutes to get rid of any cell debris. The collected supernatant would contain the aptamers that are not binding to the negative cells, this way aptamers that are bound to the receptor are concentrated. This selection process was done for 10 rounds, where every collected pool was then purified and amplified using PCR before being used for the next round of selection. Figure 1 shows a schematic of the selection process.

After every round of selection, the collected pools went through two different methods of purification and polymerase chain reaction amplification processes

3.5 Polymerase Chain Reaction

3.5 a Method 1: LATE-PCR

Polymerase chain reaction was performed to amplify the aptamer sequences in each of the collected pools after the selection processes. Linear after exponential amplification was used as the first method of amplification, in order to generate ssDNA aptamer sequences. Firstly, the concentration of the obtained aptamer pool was obtained using NanoDrop-2000 UV Vis spectrophotometer (Thermo-Scientific). Consequently, 50 ng of aptamers was used for every PCR reaction accordingly. The PCR reaction mixture contained 1X GC buffer supplemented with MgCl$_2$ of 1.5mM, 0.2mM dNTP (KAPA biosystems), forward primer at 1uM (5'-FAM-CTC CTC TGA CTG TAA CCA CG-3'), reverse primer at 0.04 uM (5'-GGC TTC TGG ACT ACC TAT GC-3') (Integrated DNA Technology), and 0.04U/ul KAPA2G polymerase (KAPA biosystems). The amplification was performed using the following protocol: preheating for 2 minutes at 95°C, 30 cycles of 10 seconds at 95°C, 20 seconds at 56°C and 10 seconds at 72°C,

and finally hold at 4°C. This amplification cycle will favour the production of ssDNA aptamers by the end of the amplification process. The amplification products tested for the presence of aptamers by running on a 3% agarose gel (sigma Aldrich) at 150 V.

The amplified pools were then purified by using a 30 kDa cut-off filter (Nanosep, PALL). The amplification products were added on top of the filter, and then it was centrifuged at 3800 xg for 10 minutes at room temperature. Following the centrifugation the filter was washed with equal volume cation-rich PBS, and centrifuged for another 10 minutes. Finally, 50ul of PBS were added on top of the filter where the aptamers are retained and collected. The concentrations were then obtained using NanoDrop-2000 UV-Vis spectrophotometer. The collected pool of aptamers was used for the following round of selection.

3.5 b Method 2: Lambda Exo-nuclease Digestion

The obtained pool of aptamers from the selection process (negative selection) was concentrated to 50 ul using 3K 2 amicon ultra filter (EMD Millipore). The products were then ran on a 1% agarose gel electrophoresis at 150 V and the aptamer band was extracted from the gel and purified using the ultra free-DA DNA extraction from agarose, (EMD Millipore). The concentration of the aptamers was obtained using the NanoDrop-2000 UV-Vis Spectrophotometer (thermo scientific). A polymerase chain reaction was then performed to concentrate the aptamers in the pool. The PCR reaction contained 50 ng of DNA from the aptamer pool. The reaction mixture constituted of 1X KAPA GC buffer with 1.5mM $MgCl_2$. 5uM dNTPs, 0.02 U/ul KAPA2G Robust Hot Start DNA Polymerase (Kapa biosystems). 5uM of the forward primer 5' CY5- CTC CTC TGA CTG TAA CCA CG-3' was used (Integrated DNA Technology), and 5uM of the phosphate labelled reverse primer 5'phosphate group- GGC TTC TGG ACT ACC TAT GC-3' was used in the reaction (Integrated DNA Technology). The PCR

program used for the reaction was as the following: Heating for 2 minutes at 95°C followed by 35 cycles of 96°C for 10 seconds, 56°C for 20 seconds of annealing, 72^{0}C for 10 seconds and finally the temperature was held at 4°C. After the polymerase chain reaction, 5 ul of the product was run on 3% agarose gel to confirm the amplification of the aptamers. The gel was ran at 150V for 20 minutes. Following the amplification, generation of ssDNA aptamers was done using exonuclease digestion. The PCR product was digested using Lambda exonuclease 1U/ml (New England Biolabs, Inc) for 2 hours at 37°C. Once the digestion was complete, the reaction mixture was heated at 80°C for 10 minutes to deactivate the exonuclease enzyme activity. The obtained pool of ssDNA aptamers was then diluted in 1ml of cation-containing PBS and utilized for the following round of selection.

3.6 Verification of Aptamer Binding using Flow Cytometry Binding assays

Once the selections were complete, the obtained aptamer pools were heated to 95°C for 10 minutes and then chilled on ice for 5 minutes. Meanwhile, cells, both CD44+ 293T and CD44- 293T cells were washed with cation-free PBS twice and scrapped off using a cell scrapper. The cells were collected in falcon tubes containing 5 ml of Cation-free PBS and centrifuged at 180 xg for 5 minutes. Consequently, the supernatant was aspirated and the pellets were re-suspended in 1 ml cation-containing PBS. The cells were the counted using Millipore Muse Cell Analyzer. Each sample for the flow cytometry assay contained 100,000 viable cells. Meanwhile, the chilled aptamer pools were added to each cell sample to a final concentration of 200nM. Therefore, each aptamer pool was incubated with CD44+ 293T and CD44- 293T as a control. As well both cell lines were incubated with the initial Harvard aptamer library (non-selected) as a control. The incubation was performed at room temperature for an hour. Following the incubation, the cells were then washed twice with cation containing PBS and the final volume was brought down to

500 ul. Unstained cells were used as a negative control/ the aptamer binding assays for pools generated by LATE-PCR were anaylzed using Guava EasyCyte flow cytometer, while assays for pools generate via exonuclease digestion was done using Beckman Coulter FC500 flow cytometer.

4-Results

4.1 Verification of CD44 expression post Lentivirus Transfection (conducted with the help of Shahrokh Ghoboloo)

Affinity assays were performed using flow cytometry to assess the expression of CD44 receptor post Lenti-virus transfection. Transfection of 293T cells with pLVX-TRE3G sub-cloned with the gene of interest: CD44. The transfection of pLVX-TRE3G-CD44 was done simultaneously with a regulator plasmid, pLVX-tet3G expressing a transactivator molecule that controls the expression of the response vector controlling CD44 expression by binding to its promoter. The expression of the transactivator molecule is controlled by the addition of doxcycline. Figure 2, shows the affinity assay, where Allophycocyanin (APC) labelled mouse anti-human monoclonal CD44 antibody is used to assess the expression of CD44. Unstained CD44- 293T and stained CD44- 293T cells were used as a negative control. Furthermore, unstained CD44+ is used as a control as well, however, stained CD44+ 293T cells were used for the assessment of the expression of CD44 and the success of the experiment.

In figure 2 a, the histogram does not represent a shift in fluorescence between the unstained and stained CD44- 293T cells. However, in figure 2 b, there is a distinct shift in fluorescence upon the addition of the antibody to CD44+ 293T cells (stained), however, unstained Cells did not shift in fluorescence.
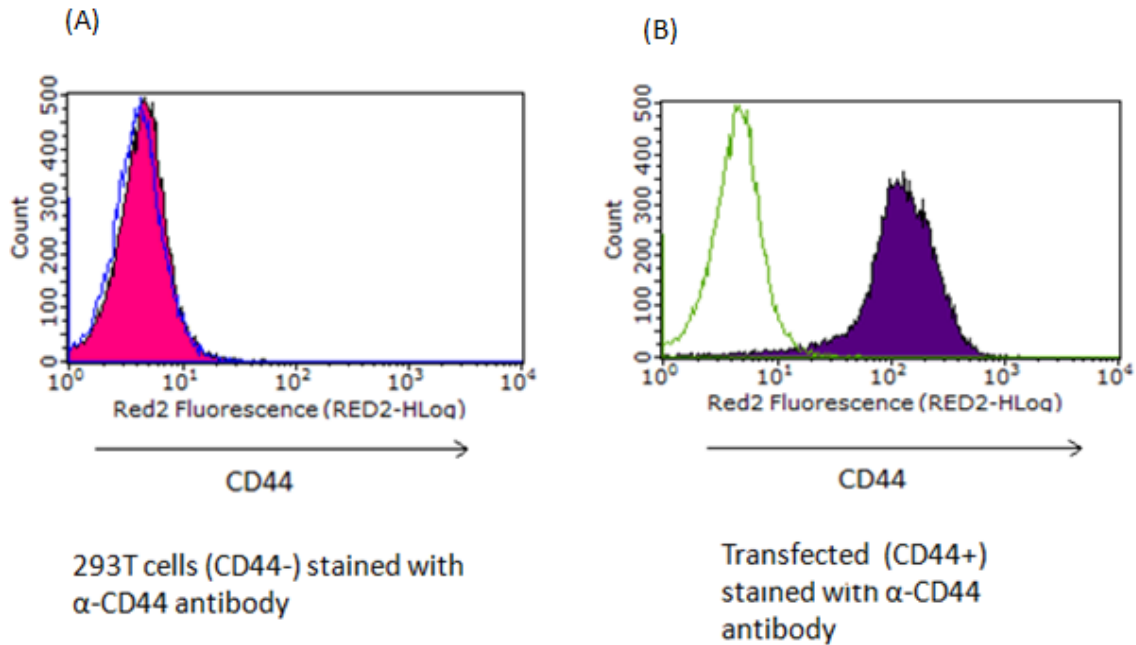
Figure 2. Verification of CD44 expression post lentivirus transfection of PLVX-TRE3G-CD44 viral vector of 293T cell line. Lentiviral transfection was performed to produce CD44+ 293T cell line. (A) Represents 293T cell line not expressing CD44 receptor both unstained and stained with allophycocyanin (APC) labelled mouse anti-human monoclonal CD44 antibody. The blue peak represents the unstained CD44- 293T cells, while the pink filled peak represents stained CD44- 293T. While, (B) represents the CD44+ 293T cell line both stained and unstained with allophycocyanin (APC) labelled mouse anti-human monoclonal CD44 antibody. The green peak represent CD44+ 293T cells unstained, while purple-filled peak represents CD44+ T293 cell line stained with antibody. Stained cells were incubated at room temperature with the antibody for one hour. The concentration of the antibody used was 5ng/ul dilution of the stock.

4.2 Flow Cytometery Binding Assays for ssDNA Aptamer Pools generated using LATE-PCR (method 1)

The Binding affinity of aptamer pools generated via LATE-PCR method (all ten rounds of selection) was tested using flow cytometry binding assays. Figure 3 shows the cells incubated with Harvard library, these results are used as control for the binding affinity assays, where both CD44+ 293T cells and CD44- 293T cells were incubated with 200nM Harvard library. The fluorescence shift was compared to the equivalent cells, however, unstained with Harvard library. From figure 3, it is observable that 200nM library did not cause a significant fluorescent shift to either cell line. A positive shift in fluorescence on the flow cytometry histogram indicates an increase in the binding affinity of the FAM-labelled aptamers to the cells. Figure 4 represents binding assays for pools 1-6 generated via cell-SELEX method, where the polymerase chain reaction used is LATE-PCR for generation of single stranded aptamer sequences. Figure 4 shows six different histograms, each assessing the binding affinity of each selected/ generated aptamer pool to CD44+ 293T cells and CD44- 293T cells, compared to unstained CD44+293T cells. Each sample of 100,000 cells was incubated with 200nM of each generated pool. A shift in fluorescence in the positive direction indicates binding to the targeted cell line.

Figure 5 represents the binding affinity assays for pools 7-10. Similar to figure 4, each histogram assesses the binding affinity of each generated aptamer pool to CD44+ 293T cells and CD44- 293T cells, compared to unstained CD44+293T cells. Since the aptamers were amplified using FAM-labelled primers, the generated pools are therefore FAM-labelled. The analysis of the generated data was done using FL1 filter, since FAM excitation wavelength is at 494 nm and emission wavelength is at 519 nm.
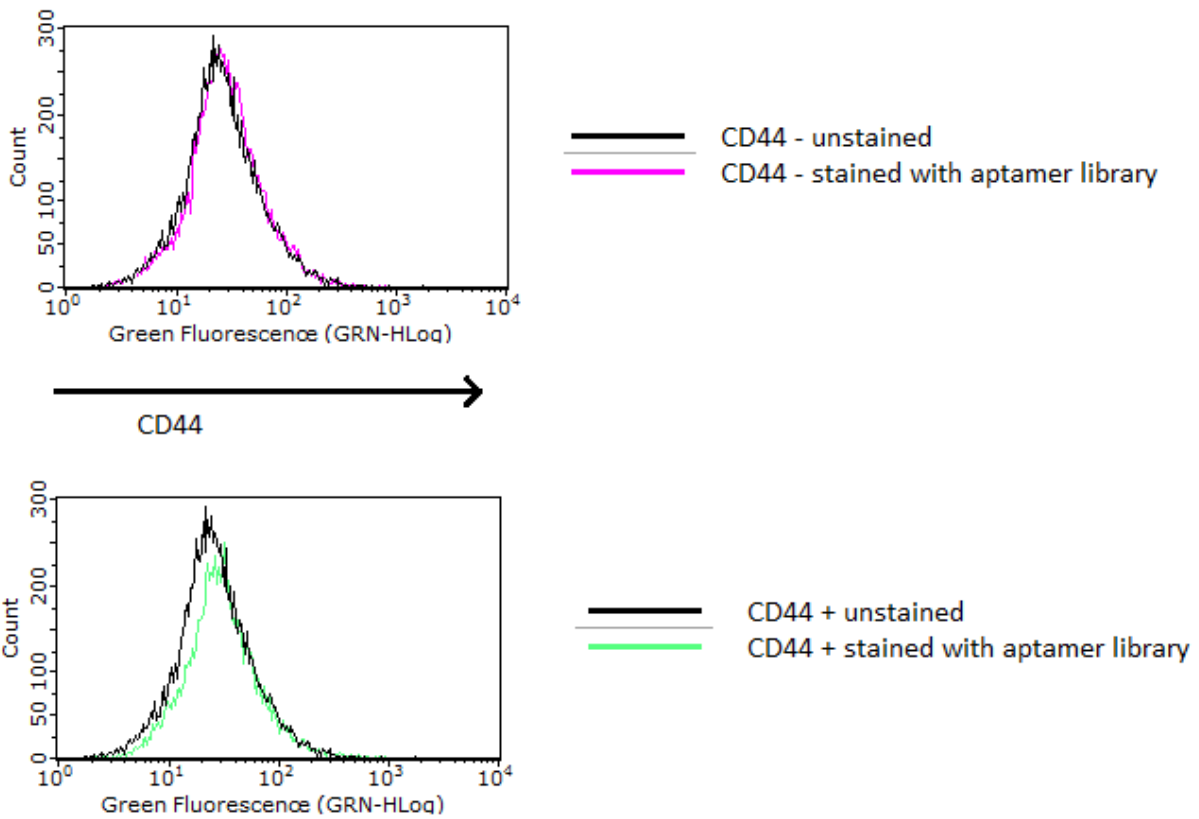
Figure 3 Flow cytometry Histogram results for affinity assay of aptamer Library to CD44+ 293T cells and CD44- 293T cells. 200nM of FAM-labelled aptamer library was incubated with CD44- 293T cells and CD44+ 293T cells for one hour at room temperature. The fluorescence of the samples was analyzed using the FL1 filter to detect the binding of the library to the corresponding cells. Unstained CD44- 293T cells and unstained CD44+ 293T cells were used as a control and baseline for the experiment.
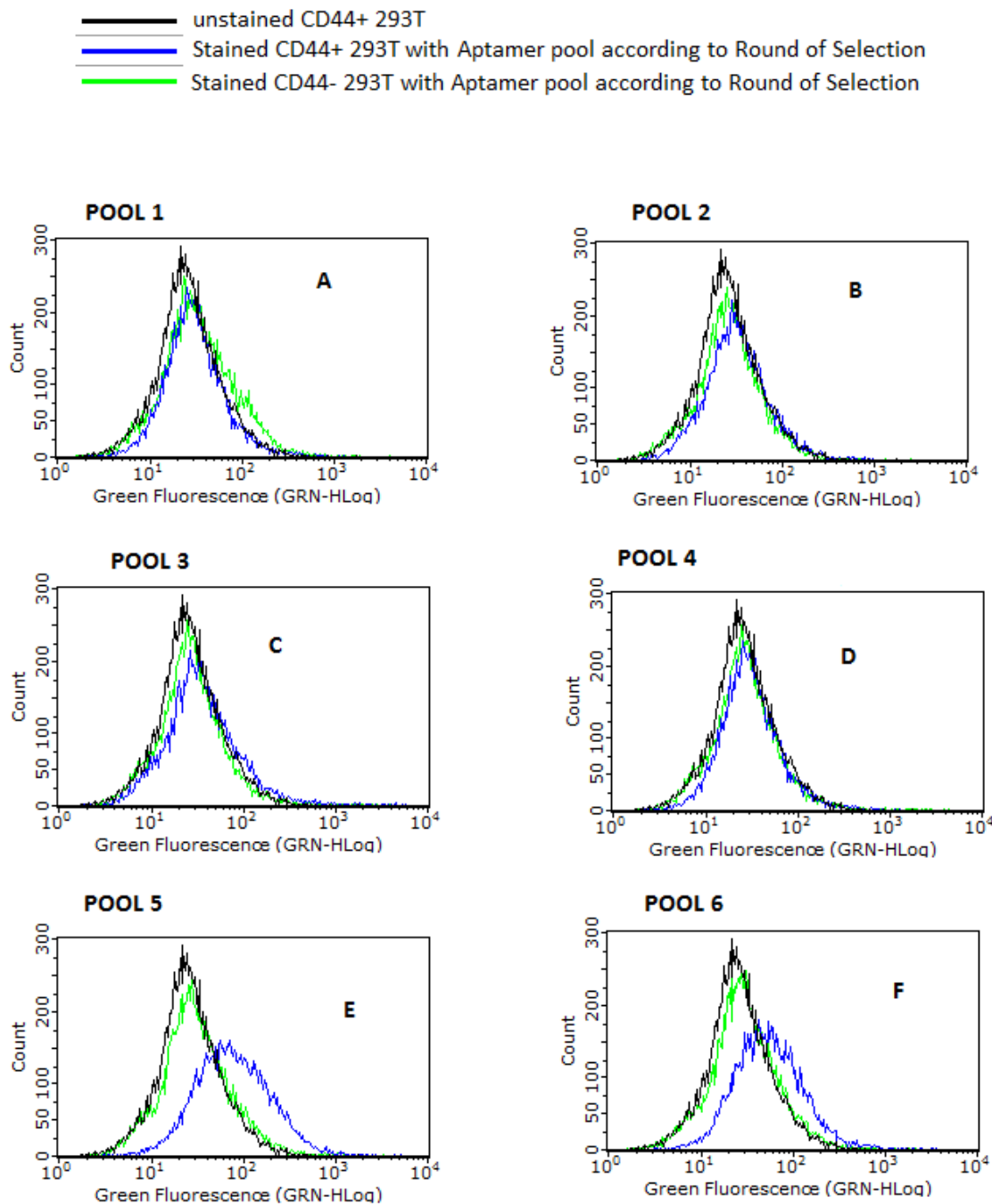
Figure 4. Aptamer binding assay for CD44 selection (method 1). In all panels, A-F, CD44+ 293T cells alone with no aptamers was used as a control baseline (the black peak). The green and blue peaks represent CD44-293T cells and CD44+ 293T cells respectively, incubated with the selected aptamer pools (1-6). Aptamers pools of final concentration 200nM were incubated with each cell line for one hour at room temperature. The data was collected using Guava EasyCyte flow cytometer, using FL1 filter since the aptamers are FAM-labelled.

Figure5. Aptamer binding assay for CD44 selection (method 1). In all panels, A-D, CD44+ 293T cells alone with no aptamers was used as a control baseline (the black peak). The green and blue peaks represent CD44-293T cells and CD44+ 293T cells respectively, incubated with the selected aptamer pools (7-10). Aptamers pools of final concentration 200nM were incubated with each cell line for one hour at room temperature. The data was collected using Guava EasyCyte flow cytometer, using FL1 filter since the aptamers are FAM-labelled.
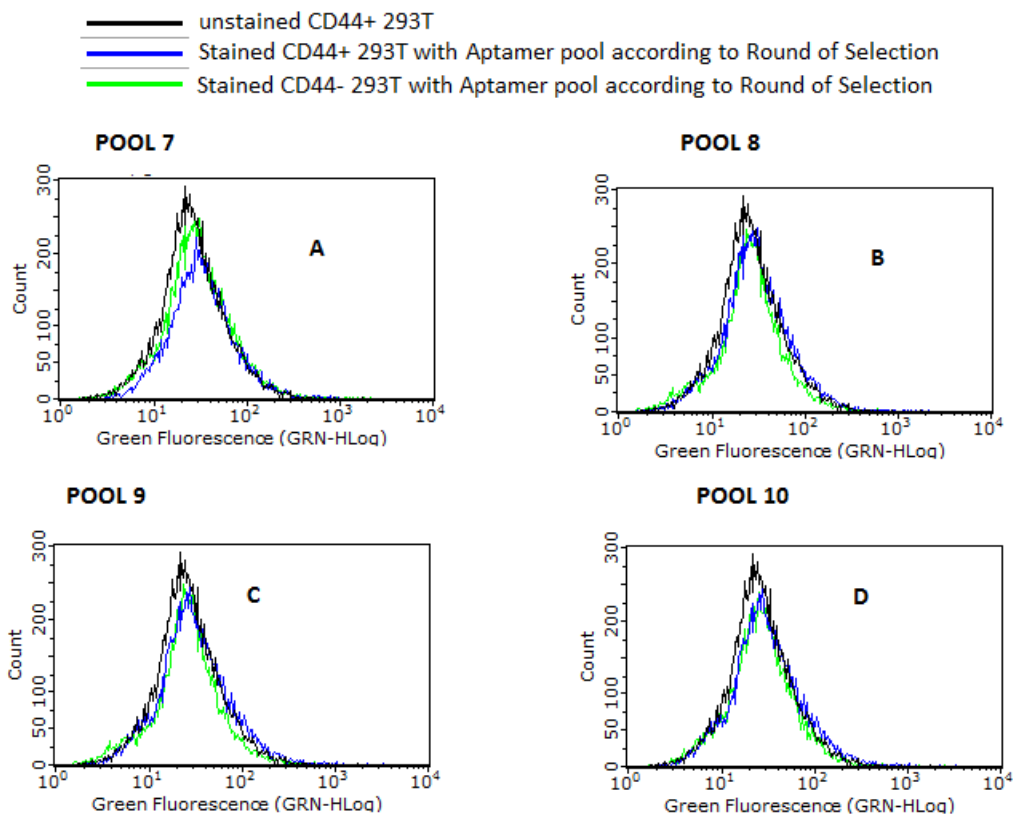
4.3 Median Fluorescence Intensity

Figure 5 represents the median fluorescent intensity (MFI) of the aptamer pools when incubated with CD44+ 293T cells and CD44- 293T cells. This value was obtained as the cells go through the FL1 filter where the binding of FAM-labelled aptamers are detected. Median fluorescent intensity is the calculated value in which 50% of the events are found and it is used to indicate the binding affinity of molecules to a cell population. It is as well used as an indicator of the central tendency of the cell population used for analysis. A higher MFI value is proportional to an increase in fluorescence, and therefore binding is achieved. From figure 5, it is evident that pool 5 and pool 6 binds most significantly to CD44+ 293T cells compared to the library binding to CD44+ 293T cells. Moreover, pool 5 and pool 6 does not show selective binding to CD44- 293T cells compared to the library control.
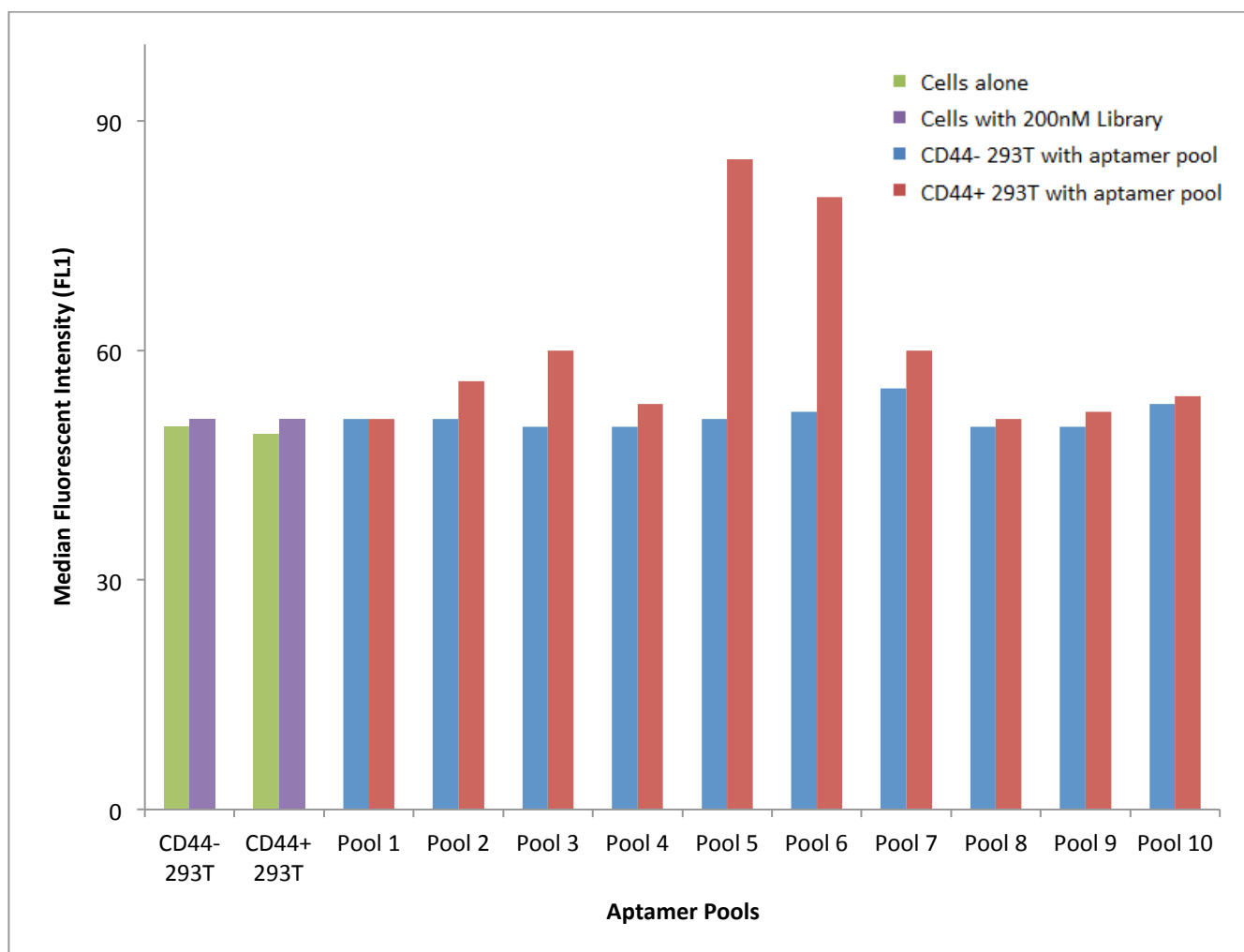
Figure 5. Median fluorescent intensity of CD44 binding assays (method 1). Median fluorescent intensity of pools 1-10 binding to CD44-293T cells and CD44+ 293T cells, represented in blue and red bars respectively. The MFI values of the aptamer pools are compared to CD44-293T and CD44+ 293T cells alone, represented with green bars. As well, it is compared to CD44-293T and CD44+ 293T incubated with 200nM Harvard library. The data was collected using Guava EasyCyte flow cytometer. Since the aptamers are FAM-labelled, the FL1 filter was used for analysis. FAM excitation wavelength is at 494 nm and emission wavelength is at 519 nm.

4.4 Flow Cytometry Binding Assays for ssDNA Aptamer Pools generated using Exonuclease Digestion (method 2)

Flow cytometry binding assays are presented in figures 6 and figure 7 for aptamer pools generated through cell-SELEX method. ssDNA aptamers were generated utilizing lambda exonuclease digestion, where the reverse primer used for polymerase chain reaction amplification is 5'-phosphate labelled. The phosphate labelled strand is selectivity digested by the exonuclease leading to the generation of ssDNA aptamers by the end of the process. The forward primer utilized for the amplification process is Cy5-labelled. The fluorescence label is used for tracking and analyzing the binding affinity of the aptamers to their target. FL4 filter was used for flow cytometry analysis, since Cy5 excitation wave length is 650 nm and emission wavelength is 670 nm.

Figure 6 represents the binding affinity to CD44+ 293T for pools 1-6. The analysis was done using Beckman Coulter FC500 flow cytometer. Unstained CD44+ 293T cells alone were used as a baseline control. Furthermore, CD44+ 293T cells stained with 200nM Harvard library was used as a control as well. A positive shift in fluorescence when a pool binds to CD44+ 293T cells but not CD44- 293T cells might indicate selective binding to CD44 receptor. Moreover, Figure 7 represents the binding affinity to CD44+ 293T cells for pools 7-10. Similar to figure 6, Unstained CD44+ 293T cells alone were used as a baseline control andCD44+ 293T cells stained with 200nM Harvard library was used as a control. No significant shift in fluorescence was observed upon addition on selected pools of aptamers.

Figure 6. Aptamer binding assay for CD44 selection (method 2). In all panels, A-F, CD44+ 293T cells alone with no aptamers was used as a control baseline (the black peak). The green and blue peaks represent CD44- 293T cells and CD44+ 293T cells respectively, incubated with the selected aptamer pools (1-6). Aptamers pools of final concentration 200nM were incubated with each cell line for one hour at room temperature. The data was collected using Beckman Coulter 500 flow cytometer, using FL4 filter since the aptamers are CY5-labelled.
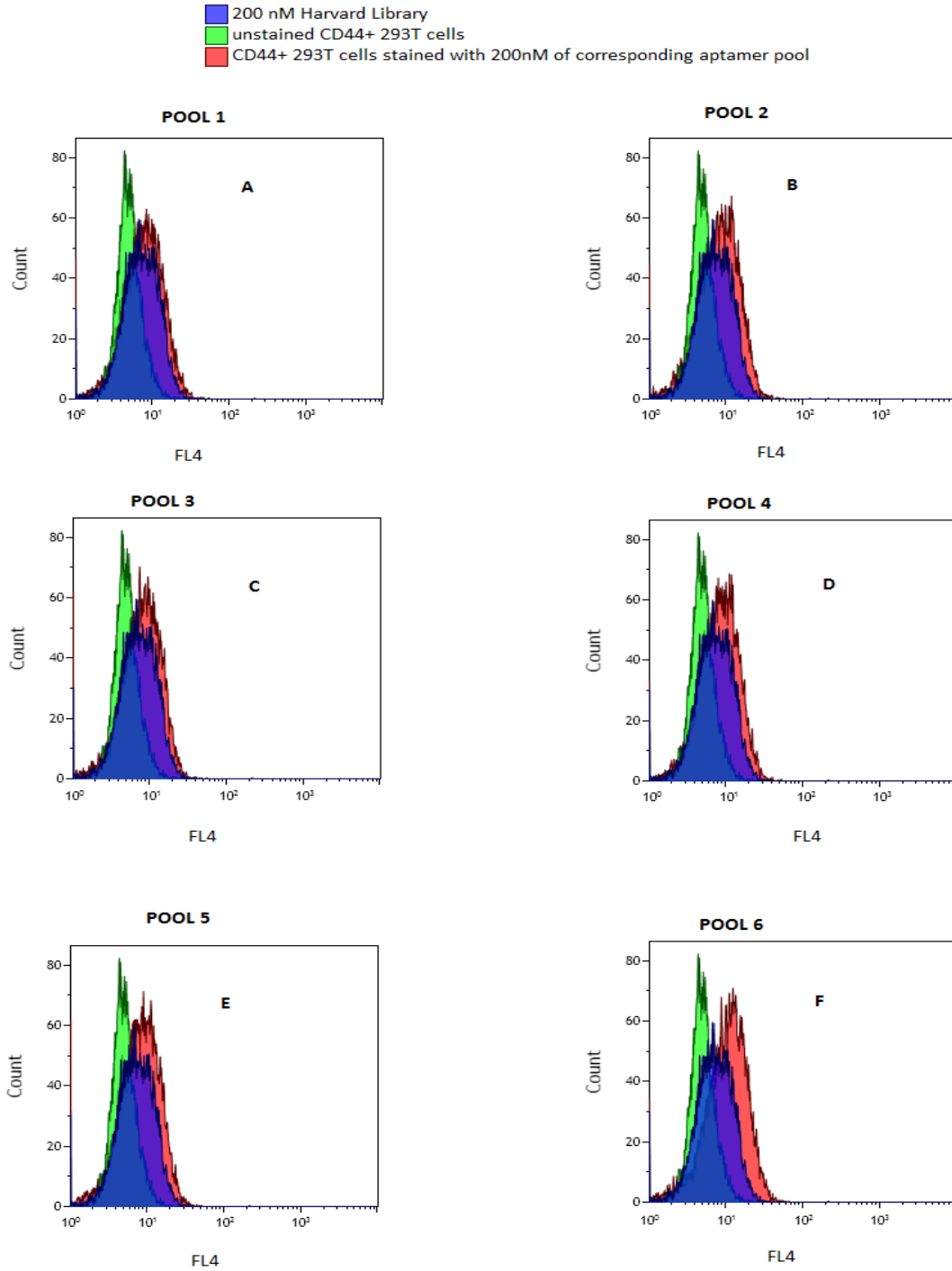
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Figure 7. Aptamer binding assay for CD44 selection (method 2). In all panels, A-F, CD44+ 293T cells alone with no aptamers was used as a control baseline (the black peak). The green and blue peaks represent CD44-293T cells and CD44+ 293T cells respectively, incubated with the selected aptamer pools (7-10). Aptamers pools of final concentration 200nM were incubated with each cell line for one hour at room temperature. The data was collected using Beckman Coulter 500 flow cytometer, using FL4 filter since the aptamers are CY5-labelled.
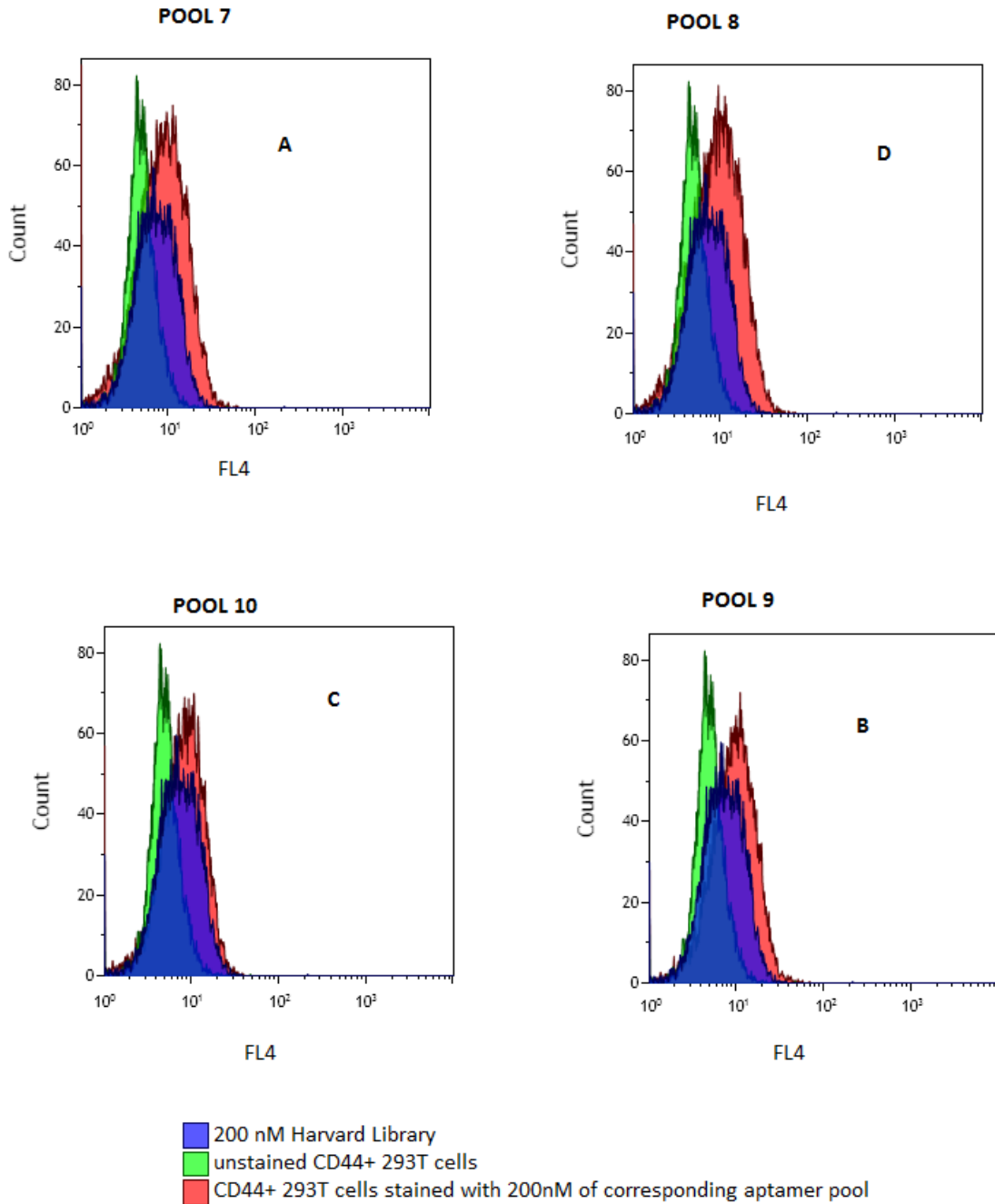
4.5 Median Fluorescence Intensity

Figure 8 represents the median fluorescent intensity (MFI) of the aptamer pools when incubated with CD44+ 293T cells and CD44- 293T cells. This value was obtained as the cells go through the FL4 filter where the binding of Cy5-labelled aptamers are detected. Median fluorescent intensity is the calculated value in which 50% of the events are found and it is used to indicate the binding affinity of molecules to a cell population. It is as well used as an indicator of the central tendency of the cell population used for analysis. A higher MFI value is proportional to an increase in fluorescence, and therefore binding is achieved. From figure 8, it is evident that there is no selective binding to CD44+ 293T cells when compared to the binding of the 200nM library or the binding of the aptamer pools to the CD44- 293T cells.
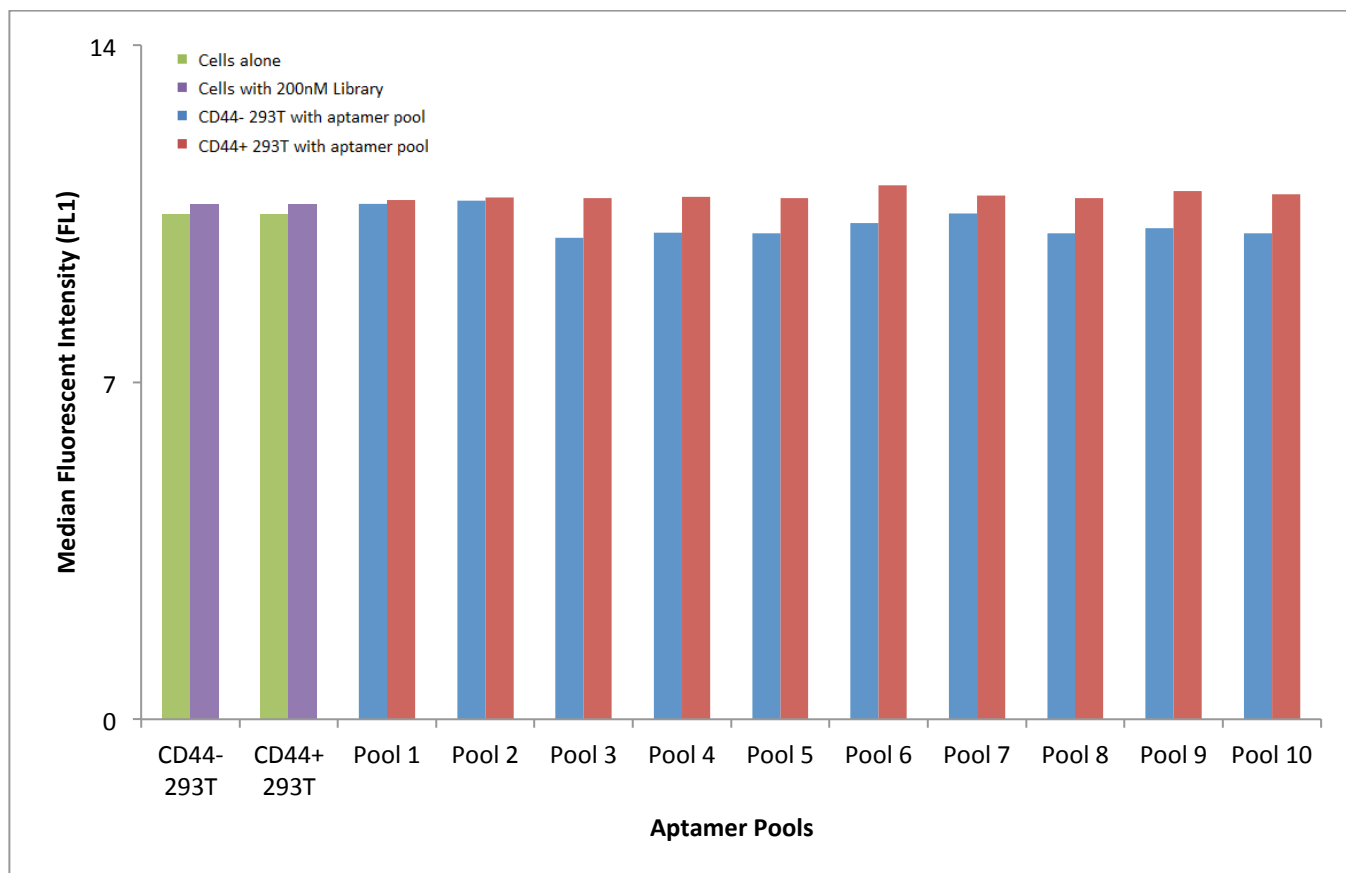
Figure 8. Median fluorescent intensity of CD44 binding assays. Median fluorescent intensity of pools 1-10 binding to CD44-293T cells and CD44+ 293T cells, represented in blue and red bars respectively. The MFI values of the aptamer pools are compared to CD44-293T and CD44+ 293T cells alone, represented with green bars. As well, it is compared to CD44-293T and CD44+ 293T incubated with 200nM Harvard library. The data was collected using Beckman Coulter 500 flow cytometer. The ssDNA pools were generated using lambda exonuclease digestion.

5-Discussion

Cell-SELEX:

Aptamers are single stranded nucleotides that are selected and identified from large nucleic acid libraries. They are characterized by their high affinity and selectivity to their target (Klussmann at al, 2006) (Stoltenburg et al, 2007). Some of their identifiable advantages are their ability to withstand reducing conditions as well as high temperatures, and thus they withstand denaturation in comparison to their protein counterparts, antibodies. Furthermore, they are easy and more economical to produce compared to antibodies. They have a wide range of application in medical science, industry and molecular biology, which make them very desirable molecules and extensively explored (Klussmann at al, 2006) (Stoltenburg et al, 2007).

In this project, the main purpose was to select ssDNA aptamers specifically to CD44 cell adhesion receptor, and optimize an *in vitro* selection method that would allow systemic and more efficient selection towards the target on live cells. Aptamers selected to CD44 have a potential to be used as a probe for visualization and detection tool that characterizes the expression level and pattern of that marker on cancer stem cells population. Since the establishment of the initial SELEX method in 1990, many modifications have been made to allow selection to specific targets (Ellington et al,1990). This SELEX method have been utilized extensively for selection of aptamers for highly purified protein targets, however, an emerged method known as Cell-SELEX emerged from the typical SELEX method. Cell-SELEX allows the selection of aptamers towards a target on a cell surface; this method is used more extensively for aptamer selection towards more complex and heterogeneous targets. Initially this method has been used and successfully selects aptamers to variety of complex targets. For example, identification of

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aptamers against red blood cells ghosts (Morris et al, 1998), as well as live African typanosomes (Homann and Goringer, 1999).

In this research, Cell-Selex was combined with a negative selection step, where elimination of aptamers binding to variants other than the target are eliminated, and aptamers binding to the target of interest are generated and concentrated.

This project attempted to generate a Cell-SELEX method that will allow selecting aptamers to CD44 utilizing two cell lines that are identical, however, the only difference between the two is that one expresses the target receptor of interest, while the other cell line lacks endogenous expression of the target receptor. The generation of the cell line was done using Lentiviral transfection method.

Lentivirus Transfection:

For this project, lenti-viral transfection was used to generate a cell line that highly expresses CD44 receptor. Those cells were used for the positive selection step of the cell-SELEX process. Lentiviral infection has multiple advantages when compared to other gene therapy methods. Firstly, this method possesses high efficiency infection rate in both dividing and non-dividing cells, they possess long term stability of the transgene, and also have low immunogenicity. Lentiviral vectors are also efficient and are easy to control in term of controlling the expression of the target transgene.

In this project a lentiviral response vector was subcloned with the CD44 gene of interest and used to transfect 293T cells. The idea behind that is to generate two identical cell lines that exhibit the same characteristics however; the only difference is the expression of the target receptor. By generation of those two cell line we were able to perform a selection method that

constitutes a positive selection followed by a negative selection process, and the aptamers collected from each round would be more specific to the target specifically, in this case CD44 (figure 1).

From figure 2, we verified the success of expression of CD44 on the cell surface through a flow cytometry affinity assay. APC labelled monoclonal antibody was used to assess the expression level of CD44 in the 293T transfected cells. These cells were treated with doxcycline post transfection to allow the expression of the response vector. CD44+ 293T cells upon the staining with the antibody, did exhibit a shift in fluorescence, unlike CD44- 293T cells, which were used as a negative control, did not show a shift upon staining with the anti-CD44 antibody. In conclusion, this experiment shows the success in creating a 293T cell line that expresses CD44 adhesion receptor, since a shift of expression in the positive direction (using FL4 filter).

Lentivirus transfection is a method that would allow performing the selection process directly on the culture plate instead of using purified protein as a target for selection, since the negative selection step would not be possible in this case. Performing selection directly on the culture plate maintains cells in their natural environment and protein targets in their native conformation, also selection would lack the destructive effects of enzymatic dissociation. Having aptamers that can bind proteins in their native state is important, and therefore would represent the natural folding structure and the possible distribution of the target(Sefah et al, 2010). All post translational modification are therefore accounted for and kept intact (Sefah et al, 2010). The selected aptamer will therefore have affinity to the possible conformation that would be found naturally, instead of the pure extracted protein. However, a described issue that could face selection directly on a culture dish/ cells in their natural environment, is the idea that cell surface receptors typically carry a net negative charge, as well as the binding aptamer in this case (since

its polyanionic) will carry a net negative charge, leading to a repulsion force between the two molecules (Sefah et al, 2010). This could interfere with the binding affinity of the aptamers to the receptor of interest. However, it is believed that the structural binding (structure interaction related binding) between the protein and the selected aptamers will be stronger than the present repulsion force (Sefah et al, 2010).

CELL-SELEX utilizing LATE-PCR amplification of aptamers (method 1)

Apatmer selection is a way of inducing artificial evolutionary pressure to select aptamers among many that would specifically bind with high affinity and selectivity to a specific target on a cell (shanguan, 2006). The overall purpose of the project is to find ssDNA apatmer sequences that would bind to CD44 efficiently. The utilized method is tailored to find high binders while minimize the usage of chemical reagents that would destroy the cell surface receptors present on the cell. Throughout the selection processes, the cells were never detached and the selection was done on the culture plate itself. Non-enzymatic dissociation buffers and cell scrapping was used to detach the cells when necessary. This allowed us to maintain the structure of the target receptor, as using trypsin to detach the cells, will ultimately cause the alteration of our target receptor.

Another interesting aspect of the selection process is the amplification process used for the polymerase chain reaction. As it is known, aptamers used for the selection process are ssDNA aptamers, while conventional PCR reaction leads to the production of double stranded DNA products. Using usual asymmetrical PCR is also inefficient and not easy to optimize, due to the fact that the concentration of one of the primers lowers its melting temperature below the annealing temperature of the reaction (Aquiles et al, 2003). Linear after exponential PCR

(LATE-PCR) is similar in efficiency as symmetric PCR while leading to the production of single stranded products instead of double stranded products that are produced by the symmetric counterpart. It possesses many favourable characteristics such as generating single stranded products with predictable kinetics with predictable kinetics beyond the exponential phase (Aquiles et al, 2003); also it possesses an increase in signal strength by 80%-250% compared to the conventional symmetric PCR. All these characteristics made the method more appealing to use in this project. For the first method of Cell SELEX used in this project, generation of ssDNA aptamers was performed using this described method.

Figure 4 represents the flow cytometry binding assays obtained for the selection processes. Apatmer selection was initiated by incubating the amplified FAM-labelled Harvard library with the lentiviral transfected cell line expressing the receptor of interest; CD44+ 293T. Following the positive selection process, the binding sequences were extracted and incubated with CD44- 293T cell line that lacks the expression of the receptor of interest. Once the incubation was done, the unbound sequences were then obtained and amplified. The amplification process was LATE-PCR, to ensure the production of ssDNA aptamers. This process was performed for 10 rounds of selection. Through figure 4, it is evident that pools 5 and 6 show a shift in fluorescence. This shift indicates binding to the aptamers to the cell surface of CD44+ 293T cells, and since there was no shift in fluorescence observed when the same pools were tested against CD44- 293T cells, it can be proposed that the binding is selective to the cell surface receptor; CD44. Even though there is binding observed in these two pools, the shift is not very significant compared to the binding of the Harvard library to cells (which was used as a control). Pool 5 and pool 6 were cloned and sent for sequencing. Due to lack of time, we were not able to test the binding of the obtained sequencing and determine their binding affinity toward the CD44+ 293T cells.

Harvard library was used for the selection process because it is a GC rich aptamer library. It has been illustrated that GC aptamer library provides a stronger binding affinity, since guanine residues are important for formation of tertiary structures, as they create three hydrogen bonds upon binding with cysteine, creating relatively stronger structures. Additionally GC residues for G-quadruplexes that help facilitate in aptamer binding (Tucker et al. 2012). Therefore, the choice of using Harvard library for the selection process is ideal since the GC content of the library exceeds 65%.

Figure 5 illustrates the binding affinity assays for pools 7 to 10. It seems that the aptamers were lost after pool 6 and the enrichment of the pools with binding sequences were lost. Theoretically, the binding affinity to the positive cell line (CD44+ 293T) should increase as the rounds of selection increase, till it reaches the most optimal binding. However, beyond pool 6, a sharp decline in fluorescence is observed, where starting from pool 7, the binding affinity of the pools to both CD44+ and CD44- cell line is almost identical and therefore, we conclude the loss of the binding aptamers. This phenomenon can be attributed to multiple events that might have lead to this decrease in fluorescence. Damaging of the target CD44 on the adherent cell line while preparing the samples for flow cytometry assay can contribute to the loss of binding apatmer sequences. Scrapping the cells can lead to mechanical stress while dissociating the cells using non-enzymatic dissociation buffer. Trying to incubate the cells for more than 10 minutes in non-enzymatic dissociation buffer prior to scrapping the cells off, can potentially decrease the damage associated. Also using trypsin for less than 30 seconds, prior to scrapping can decrease the mechanical stress; however, there is still the issue of alteration due to enzymatic manipulation.

Cell-SELEX utilizing Lambda exonuclease Digestion (Method 2)

The second selection method in this project is identical to the first method, except that the amplification process used for generation of ssDNA apatmers is different. Lambda exonuclease digestion is used instead of LATE-PCR for this method. The reverse primer is 5'-phosphate group labelled. Conventional polymerase chain reaction is first used to produce double stranded products. Following the amplification, lambda exonuclease is used to digest the amplified double-stranded DNA into single-strand fragments, where digestion occurs to the phosphate labelled strand. This process allows fast and efficient way to produce ssDNA aptamers.

Figure 6 represents the binding affinity assays for pools 1-6 created by the second amplification method. The aptamer pools in this case are Cy5-labelled and are therefore FL4 filter was used for flow cytometry analysis, since Cy5 excitation wave length is 650 nm and emission wavelength is 670 nm. Pools 1-6 do not show a significant positive shift in fluorescence compared to the 200nM Harvard library control shown. Also, figure 7 (representing pools 7-10) no increase in fluorescence is observed when the pools are tested for binding with CD44+ 293T cells. Figure 8 illustrates the median fluorescence intensity illustrated for pools 1-10 representing the binding affinity to both CD44+293T cells as well their binding affinity to CD44- 293T cells. Unfortunately, the MFI is almost the same for each cell line when incubated with each of the selected pools. Therefore, it can be concluded that no selective binding was observed. Theoretically, after 10 rounds of Cell-SELEX, there should be an observed increase in fluorescence when incubated with CD44+ 293T, since there should be an increasing concentration of strongly binding aptamers to the target and elimination of weakly bound aptamers or aptamers that binds selectively to CD44- 293T cells. In this case, by increasing the rounds of selection to more that 10 rounds can result in an observable shift in fluorescence in favour of the CD44+ cells. Perhaps, when preparing the samples for the flow cytometry assay,

too much mechanical stress leads to alteration of the receptor when scrapping off the cell (CD44 is an adhesion receptor). Furthermore, lambda exonuclease digestion used in this experiment could be inefficient in generating ssDNA aptamers. It is well established that generation of ssDNA molecules plays a very important role in SELEX. Therefore the purity and the yield of the process are very important (Avci-Adali et al. 2009). Perhaps the yield of ssDNA aptamers was not very high and having dsDNA aptamers will not result in efficient binding after all, leading to the loss of aptamers during the process. Even though, theoretically lambda exonuclease digestion does provide a high ssDNA yield, the issue is that we did not eliminate the presence of the enzyme beyond its heat denaturation after digestion. The presence of lambda exonuclease acts as an additional SELEX target molecule and consequently interferes with the selection process (Avci-Adali et al. 2009). A possible way to eliminate this problem is treatment of the aptamer pool with phenol/chloroform extraction and subsequent ethanol precipitation to eliminate the enzyme from the aptamer pool. However, even though this step successfully eliminates the exonuclease, it also leads to the loss of approximately 40% of the generated ssDNA amount(Avci-Adali et al. 2009).

Literature Selected CD44 Apatmers

According to a primary research article, some aptamers have been previously selected toward CD44 as a purified protein, not in cell surface environment. According to somasundern et al. (2010), they have selected aptamers to the target protein through a selection process involving a library of aptamers that is thiolated on the 5' side of every adenine residue. This was based on previous evidence suggesting that thioloation of adenine residues increased association constant of RNA and DNA to coat protein by greater than 10 fold, whereas thiolation of other residues did not have any effect (Milligan and Uhlenbeck, 1988). Other evidence suggested that thiolation of

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aptamers did not have an effect in improving the binding affinity, rather it only have effect in improving aptamer resistance to nuclease activity and degradation prevention (Sefah et al. 2010).

Furthermore, according to the available literature, RNA aptamers have been also selected toward full length human recombinant CD44. The selection process was done towards the purified protein, not on cell surface environment, using normal SELEX method not Cell-SELEX. The library used for the selection process was 2'-F-pyrimidine modified RNA library with a complexity of around 10^{14} different molecules. 2'-F-pyrimidine modified nucleotides is believed to provide more stability, providing better binding affinity upon selection (Ababneh et al. 2013).

Future Directions

Based on the collected data, the flow cytometry binding assays must be repeated with higher aptamer pool concentrations. For the purposes of the previous analysis, 200 nM concentrations of the obtained pools were used for the analysis, perhaps increasing the concentration, would provide better results. Furthermore, increasing the rounds of selection could possibly provide more selective aptamers. Also, using modified aptamer library, for example 2'-F-pyrimidine library or 5'-adenine thiolated aptamers could provide better stability and improve nuclease resistance. Furthermore, using biotin labelled aptamer library and streptavidin beads to generate ssDNA aptamers showed more promising results for generation of single stranded nucleotide pools (Sefah et al. 2010). As for future direction, pools 5 and 6 from method 1 (LATE-PCR) will be cloned and sequenced, allowing further analysis and assessment of the apatmers. Obtained aptamers from these pools will be assessed for their binding using flow cytometry binding assays. Overall, further assessments should be conducted to solidify and elucidate the results.

6-Conclusion

CD44 is an important marker for certain populations of cancer stem cells, and has been implicated in tumorgeneity and increased invasiveness of cancer. The cancer stem cell hypothesis was generated based on several experimental approaches demonstrating the ability of this subpopulation of cells to initiate and maintain tumour regardless of the conventional anti-cancer therapies available. From there, a development of methods to isolate cancer stem cells is essential and needed in order to better understand their behaviour and allow for further studies. The isolation of those cells is mainly controlled by the ability to identify specific protein markers that are expressed on their surface. Although CD44 has been identified to be highly expressed on cancer stem cells, total exclusiveness of expression not achieved, as CD44 is also expressed (in lower levels) on other cells. This project aimed on using Cell-SELEX aptamer selection method to isolate aptamers for the marker CD44. Aptamers can act as a method to exploit CD44-hylauronic acid interactions for targeted drug delivery for cancer stem cells as well as inhibiting this interaction leading to inhibition of metastasis, invasion and cancer recurrence. From this project, pools 5 and 6 generated using LATE pcr showed preferential binding towards CD44+ 293T cells, which suggests possible aptamers for CD44 receptor. Overall, the conditions used for this project did not prove to be highly successful as further optimization of the protocol needs to be conducted. Suggestions for improved selection processes include the usage of higher aptamer pool concentration when performing the affinity binding assays. Furthermore, using biotin labelled aptamer library and streptavidin beads to generate ssDNA aptamers showed more promising results for generation of single stranded nucleotide pools. Finally, using a modified initial library could increase and strengthen the aptamers' ability to bind and allow for nuclease resistance.

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