

DEVELOPMENT OF DNA APTAMERS SPECIFIC FOR NON-SMALL CELL LUNG CANCER

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Doctoral Dissertation
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RAZVOJ DNA APTAMER SPECIFIČNIH ZA CELICE
NEDROBNOCELIČNEGA PLJUČNEGA RAKA

Doktorska disertacija

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To my parents and the best sister in the world.

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Abstract

Lung cancer has the one of the highest incidence and death rate among all types of cancer. When lung cancer patients are diagnosed at the late stage, it is rarely curable; thus, early diagnosis is one way to improve lung cancer patients' survival rate. Detection of circulating lung cancer cells would represent an improved diagnostic tool to indicate metastasising tumours at an early stage, contributing to better prognosis of the disease. To address this issue, this dissertation aimed to design aptamers for lung cancer cells, as alternatives to antibodies for detection of circulating cancer cells with stem cells characteristics as a novel tool for diagnosis and following the progression of lung cancer.

Aptamers are single stranded DNA or RNA molecules, capable of forming three-dimensional (3D) structures fitting the target molecules. Aptamers are chemically synthesized and have long shelf life and high specificity and affinity for the target proteins and/or cells. They are more robust for different types of modification and conjugation compared with antibodies of the same specificity. With the Cell-based Systematic Evolution of Ligands by EXponential enrichment (Cell-based SELEX) technique, aptamers can also be readily developed for any types of cells of interest. The resulting aptamers recognize their target molecules in their native state in the original cellular context. Our approach focuses on aptamer development for whole non-small cell lung cancer (NSCLC) cells expressing the stem cell surface marker CD90.

The human adenocarcinoma cell line A549 was used as a target in seven SELEX rounds. The last round included selection for a cell population positive for CD90 expression.

We identified the aptamer A155_18, which had good characteristics for binding to cell line A459 in terms of high affinity, consensus motifs and structural stability. The final selection analysis was based on applying three independent bioinformatics approaches in parallel, two of them resulting in selecting the same candidates with the best binding characteristics.

This aptamer was then compared with the reported A459 cell-binding aptamer S6, which served as positive control. The selected pool was used for sequencing and subjected to three independent bioinformatics analyses. The calculated dendrogram showed that aptamers A155_18 and the S6 have a close structural relationship. According to one of the used sequence analysis tool, called MEME, they share two unique motifs, which are not present in other identified sequences.

Noteworthy, as A459 cells were binding both, aptamer A155_18 and the CD90 antibody, a partial stemness characteristics of the primary A459 cell line, or more likely indicates on the presence of stem cell-like subpopulation within the primary cultured non-small cell lung cancer cell line.

In conclusion, in this work we successfully developed a novel aptamer A155_18 binding to CD90-positive A549 lung cancer cells. The novelty of our approach is the in silico selection of aptamers with emphasis on bioinformatics-based prediction of secondary structures and theoretical physicochemical properties, enabling the direct selection of the most appropriate oligonucleotide aptamer. Besides early diagnosis, this aptamer could also be used in search for identification of other target proteins on NSCLC cell surface and potentially contribute to biomarker discovery for lung cancer.

Povzetek

Pljučni rak je med najbolj pogostimi in smrtonosnimi oblikami rakavih obolenj. Le redko je ozdravljiv, saj je pogosto odkrit v pozni fazi, zato bi zgodnja diagnoza pripomogla k daljšemu življenju bolnikov. Zaznavanje in določevanje krožečih celic pljučnega raka je pomembno za zgodnjo diagnozo oziroma za odkrivanje metastazirajočega tumorja, kar bi lahko izboljšalo prognozo bolezni. V doktorskem delu smo želeli pripraviti aptamere za določanje celic pljučnega raka kot alternativo protitelesom za prepoznavanje krožečih rakavih celic z lastnostmi matičnih celic v krvi kot novo orodje za zgodnjo diagnozo in sledenje napredovanja pljučnega raka.

Aptamere so enoverižne DNK ali RNK molekule, sposobne zvitja v tridimenzionalne (3D) strukture, prilagajoče se tarčnim molekulam. Aptamere so kemijsko sintetizirane, njihova prednost je v obstojnosti in v veliki specifičnosti in selektivnosti prepoznavanja ter afiniteti do tarčnih proteinov in/ali celic. Lažje jih tudi modificiramo in konjugiramo v primerjavi s protitelesi, ki bi bila enako specifična. Z metodo celičnega SELEXa (sistematske evolucije ligandov z eksponentno obogatitvijo) lahko razvijemo aptamere proti raznim vrstam rakavih celic, pri čemer tarčne molekule ostanejo v svoji nativni obliki in v izvornem celičnem kontekstu. V našem delu smo pridobili novo aptamero, ki specifično prepozna celice nedrobno-celičnega pljučnega raka (NSCLC) in ki izraža tudi površinski označevalec matičnosti CD90.

Tarčna celica za načrtovanje aptamer v procesu pozitivne selekcije sedmih ciklov (krogov) SELEXa je bila človeška celična linija pljučnega adenokarcinoma A549. Kot negativno selekcijo smo v zadnjem krogu uporabili protitelesa proteina CD90. Po sedmih krogih selekcije smo izbrali aptamero A155_18 zaradi močne vezave na celice A549, dobro prepoznavnih strukturnih motivov in njihove stabilnosti. Tako pridobljene oligonukleotide smo sekvencirali, analizirali z različnimi bioinformatičnimi pristopi ter primerjali z že znano aptamero S6, ki smo jo uporabljali kot pozitivno kontrolo za vezavo na celice A549. Končna analiza izbora kandidatnih aptamer z najboljšimi vezavnimi lastnostmi je zatem temeljila na treh neodvisnih bioinformatičnih pristopih, ki pa so pokazali enake sekvence. Na podlagi teh podatkov smo s pomočjo konstruiranega filogenetskega drevesa prikazali, da sta naši izbrani aptameri A155_18 in S6 strukturno sorodni. S pomočjo bioinformatičnega orodja, imenovanega MEME, smo lahko dokazali, da imata aptameri dva enaka, specifična motiva, ki se ne pojavljata v ostalih sekvencah kandidatnih aptamer, izbranih v zadnjem krogu selekcije. Ker je imela primarna celična linija A549 tako afiniteto do aptamere A155_18 in se je vezala na protitelesa za CD90, lahko sklepamo, da ima ta linija tudi delne lastnosti matičnosti oziroma kaže na prisotnost subpopulacije z lastnostmi matičnosti v tej liniji nedrobnoceličnega pljučnega raka.

V doktorski disertaciji smo uspešno razvili novo aptamero A155_18, ki prepozna tarčno celično linijo pljučnega karcinoma A549, ki izraža tudi biološki označevalec matičnih celic CD90. Naš pristop je izviran v tem, da smo uporabili in silico selekcijo aptamer s poudarkom na bioinformatičnem predvidevanju sekundarne strukture in teoretičnih fizikalno-kemijskih lastnosti, kar je omogočilo neposreden izbor najbolj primerne aptamerne oligonukleotida. Poleg klinične uporabe v diagnozi pljučnega adenokarcinoma je aptamera primerna tudi za nadaljnje odkrivanje tarčnih proteinov na površini NSCL celic in bi tako lahko prispevala k možnemu odkrivanju novih bioloških označevalcev pljučnega raka.

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Abbreviations

ABC	... ATP-binding cassette
AC	... Adenocarcinoma
ACS	... Acute coronary syndrome
ALDH1	... Aldehyde Dehydrogenase 1
ALK	... Anaplastic lymphoma kinase
AMD	... Age related macular degeneration
ASO	... Antisense oligonucleotide
ATCC	... American Type Culture Collection
BRAF	... B-Raf protein encoding gene
BRASIL	... Biopanning and rapid analysis of selective interactive ligands
BSA	... Bovine Serum Albumin
CD133	... Transmembrane glycoprotein prominin-1
CD44	... Homing cell adhesion molecule; HCAM
CD90	... Thy-1 cell surface antigen
COMPAS	... COMMon PAtternS
CSC	... Cancer stem cell
CT	... Computed tomography
CTC	... Circulating tumor cell
DMEM	... Dulbecco's Modified Eagle Medium
DMSO	... Dimethyl sulfoxide
DNA	... Deoxyribonucleic acid
EDTA	... Ethylene-diamine-tetra-acetic acid
EGFR	... Epidermal growth factor receptor
ELISA	... Enzyme-Linked Immunosorbent Assay
EMT	... Epithelial-to-mesenchymal transition
EpCAM	... Epithelial cell adhesion molecule
E-selectin	... Endothelial-leukocyte adhesion molecule 1
FACS	... Fluorescence activated cell sorting
FACS	... Fluorescence activated cell sorting
FAM	... Fluorescein amidite
FASTA	... DNA and protein sequence alignment software package
FBS	... Fetal Bovine Serum
FDA	... Food and Drug Administration
FISH	... Fluorescent in situ hybridization
FITC	... Fluorescein isothiocyanate
GPI	... Glycosylphosphatidylinositol
GUI	... Graphical user interface
HEPES	... 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2	... Receptor tyrosine-protein kinase erbB-2
HTS	... High throughput sequencing
ICAM	... Intercellular Adhesion Molecule
IgG	... Immunoglobulin G
IMRT	... Intensity-modulated radiation therapy
ISET	... Isolation by Size of Epithelial/Throphoblastic Tumor cells
Kd	... Dissociation constant
LC	... Large cell carcinoma
MEGA	... Molecular Evolutionary Genetics Analysis
MEME	... Multiple em for Motif Elucidation
MET	... Mesenchymal-to-epithelial transition
MOP-1	... Mediator of paramutation1

MRI	... Magnetic resonance imaging
mRNA	... Messenger RNA
MS	... Mass spectrometry
NFκB	... Nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	... Next generation sequencing
NSCLC	... Non-small cell lung cancer
PAGE	... Polyacrylamide gel electrophoresis
PBS	... Phosphate-buffered saline
PCR	... Polymerase chain reaction
PCR	... Polymerase chain reaction
PEG	... Polyethylene glycol
PET/CT	... Positron emission tomography-computed tomography
PGM	... Personal genome machine
PI	... Propidium iodide
RET	... RET Proto-oncogene
RFLP	... Restriction fragment length polymorphism
RFU	... Relative fluorescence unit
RNA	... Ribonucleic acid
ROS1	... Reactive oxygen species
RT-qPCR	... Real-time quantitative polymerase chain reaction
SCC	... Squamous cell carcinoma
SCF	... Stem cell factor
SCLC	... Squamous cell lung cancer
SDF-1	... Stromal cell-derived factor 1
SELEX	... Systematic evolution of ligands by exponential enrichment
SiRNA	... Small interfering RNA
SP	... Side population
ssDNA	... Single stranded DNA
STR	... Short tandem repeat
TFPI	... Anti-tissue factor inhibitor
T _m	... Melting temperature
TNF-α	... Tumor necrosis factor alpha
VCAM	... Vascular cell adhesion protein
VEGF	... Vascular endothelial growth factor
vWF	... Von Willebrand Factor
WBC	... White blood cells
WHO	... World Health Organization
Zeb	... Zinc finger E-box-binding homeobox

Chapter 1

Introduction

1.1 Lung Cancer

Lung cancer incidence and death rates have been rising during most of the twentieth century and, according to Cancer Statistics in 2017, it accounts for approximately 26 % of all cancer-related deaths (R. Siegel, 2017).

For the most aggressive and lethal forms of cancer, the identification and characterization of cancer stem cells in these malignancies may provide the best leads for the development of new and more effective therapeutics. Although lung cancer is among the most commonly lethal forms of cancer in the world, comparatively less is known about the biology of lung cancer stem cells compared to other solid tumour stem cells (Carafaro, 2002). Therefore, there is a pressing need for the development of new therapeutic agents that better manage the progression of highly aggressive lung cancer cells. However, the methods to identify and isolate self-renewing lung cancer stem cells are still being developed and the exact marker identity of human lung cancer stem cells remains controversial. This is due in part to the complexity of the disease in terms of its phenotypically diverse and regionally distinct types of neoplasia. Lung cancer is comprised of four major histological types: small cell lung cancer (SCLC) and three types of non-small cell lung cancer (NSCLC): squamous cell carcinoma (SCC), adenocarcinoma of the lung (AC), and large cell carcinoma (LC) (Figure 1.1: Histological types of lung cancer. Lung cancer is divided in two main groups: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). The latter is further comprised of adenocarcinoma, squamous cell carcinoma and large cell carcinoma. The percentage of total share and arising location is presented respectively.) (J. R. Molina, Yang, Cassivi, Schild, & Adjei, 2008).

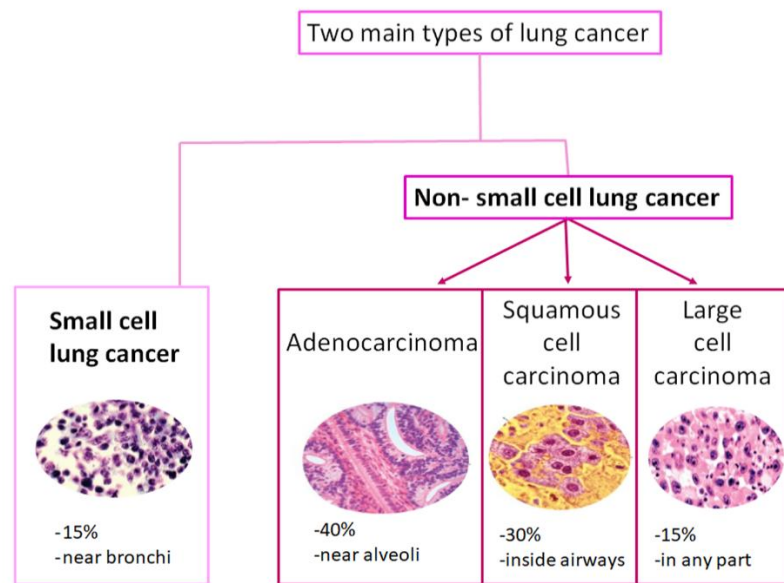


Figure 1.1: Histological types of lung cancer. Lung cancer is divided in two main groups: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). The latter is further comprised of adenocarcinoma, squamous cell carcinoma and large cell carcinoma. The percentage of total share and arising location is presented respectively.

1.1.1 Non-small lung adenocarcinoma

Non-small-cell lung cancer (NSCLC) constitutes more than 80% of all new lung cancer cases (Namba et al., 2004). Over 50 % of patients are diagnosed initially with a locally advanced or metastatic disease with worse outcomes. Currently, most patients with NSCLC are diagnosed with advanced cancer. Symptoms of metastatic cancer include weight loss, bone pain, headaches, anaemia, and paraneoplastic syndromes. The preliminary diagnosis of metastatic disease is based on symptoms, signs, and laboratory tests and it is aided by imaging (eg, PET/CT scan, brain MRI). Although surgery is regarded as the optimal treatment, only 25–30 % of patients are suitable for potentially curative resection and despite optimal surgical management, their 5-year survival rate ranges between 73 and 25 % for pathological stage Ia and IIIa, respectively (Buffoni, Vavalà, & Novello, 2016). Patients with widespread metastatic disease (stage IV) are usually candidates for systemic therapy that consists of chemotherapy, targeted therapy, or immunotherapy, depending on performance status and results from biomarker testing, clinical trials, and/or palliative treatment. Recent treatment investigation strategies has been focused on molecular features of the tumour, that are involved in either the tumour growth or mechanism of action of anticancer drugs, especially DNA repair enzymes and drug transporters. Such biomarkers can provide additional prognostic information to the existing clinic-pathological tumour staging data, and thus potentially help in patient selection for adjuvant chemotherapy.

Several biomarkers have emerged as predictive and prognostic markers for NSCLC. Predictive biomarkers include the ALK fusion oncogene, ROS1 gene rearrangements, and sensitizing EGFR mutations. Emerging biomarkers include HER2 (known also as ERBB2) and BRAF V600E mutations, RET gene rearrangements, and high-level MET amplifications. Testing for genetic alterations is recommended in patients with NSCLC because targeted therapy has been shown to decrease tumour burden and disease symptoms, and to dramatically improve the quality of life for patients. Specific targeted therapies are available for the treatment of advanced NSCLC, thus they interact with the biomarker's signalling pathways. Targeted agents, such as Erlotinib, Gefitinib, Afatinib, and Crizotinib are the first-line therapy based on larger trials (Buffoni et al., 2016; Ettinger et al., 2017).

Radiation dose is another issue with possible implications on survival since currently approved screening strategies involving low dose CT scans have a low sensitivity and very high false positive rates (> 90 %). Recent clinical studies have shown that higher doses would have

better outcome but the unanticipated results could be due to the increased toxicity associated with higher photon radiation. Therefore, the standard dose of radiation for definitive chemo-radiation remains unchanged. Technological advances in the delivery and dose optimization of radiotherapy have contributed substantially to the improved survival rates of patients. Newly emerging modalities can now employ respiration-correlated CT scans ('4D CT'), intensity-modulated radiotherapy (IMRT), and proton therapy (Blendon, Kim, & Benson, 2010; Schild, Ramalingam, & Vallieres, 2016).

However, there is need for additional biomarkers that can improve the sensitivity of low dose CT screening, particularly in patients with indeterminate pulmonary nodules. Following a diagnosis of lung cancer and stage-specific therapy, currently the only available techniques to monitor disease progression other than clinical symptoms are periodic CT scans done every 3-6 months. Earlier detection of resistance in this subgroup of patients using a minimally invasive approach could potentially serve as to aid decisions on subsequent alternative therapies targeting secondary mutations or alternative pathway activation (Kobayashi et al., 2018). Furthermore, a better non-invasive approach for serial monitoring is necessary not only for early response evaluation for targeted therapies but also to address clinical and research needs.

1.2 Circulating Tumour Cells

Emerging research in blood-based biomarkers provides new opportunities to diagnose lung cancer earlier and to assist with detection of earlier recurrence. These biomarkers include circulating tumour cells (CTCs) that have been extensively studied as prognostic and pharmacodynamics biomarkers in many cancers (Alix-Panabières & Pantel, 2013; Botteri et al., 2010; Danila, Fleisher, & Scher, 2011; Krebs et al., 2011; Nurwidya et al., 2016). CTCs are cells shed by underlying tumours that circulate in blood and lymphatic vessels and are the likely drivers of metastasis, which accounts for nearly 90% of cancer-related deaths (Bednarz-Knoll, Alix-Panabières, & Pantel, 2012). CTCs express putative stemness and mesenchymal markers, such as vimentin, N-cadherin, fibronectin, alpha-smooth muscle actin thus suggesting the undergoing epithelial- to-mesenchymal transition (Messaritakis et al., 2017). CTCs may be pre-metastatic conditioning cells, lying dormant and providing a 'pre-metastatic niche'- a tumor microenvironment for the subsequent formation of future metastases by other cancer- initiating cells. The latter is comprised by a milieu of different cell types (cancer cells, fibroblasts, stromal cells, immune cells and extracellular matrix as a reservoir of growth factors and signaling molecules). Pro-inflammatory cytokines and adhesion molecules (TNF- α , E-Selectin, P-Selectin, VCAM-1 and ICAM-1) expressed by cells of the tumor microenvironment induce tissue specific up-regulation of integrin which leads to secretion of matrix metalloproteinases and cleavage of the extracellular matrix. This subsequently results in the release of growth factors and signaling molecules that promote tumor cell growth, angiogenesis and invasion (Caixeiro et al., 2014).

Accordingly a small subset of CTCs possess cancer stem cell (CSC) characteristics based on their ability to survive in the circulation and to give rise to the metastatic lesions. CTCs may be a viable non-invasive alternative to tissue biopsies for diagnosis of lung cancers. In many patients, however, CTCs are quite low in number, and they need to be isolated from an overwhelming majority of blood cells (1 CTC: 1 billion blood cells). CTCs play a major role in the development of personalized medicine and targeted therapies. Additionally, CTCs have been proposed as surrogate biomarkers in a multitude of research areas, including the selection of neoadjuvant and adjuvant therapy, detection of recurrent disease, and as pharmacodynamics biomarkers of novel therapeutics (Gingras, Salgado, & Ignatiadis, 2015; Pantel & Alix-Panabières, 2013). They can ultimately lodge to, invade, and proliferate distant organs, initiating metastatic lesions and acquiring mutations beyond those initiated within primary tumours (Nguyen & Massagué, 2007). Therefore, detecting CTCs can be extremely valuable for cancer diagnosis in early stages and help with treatment decisions.

1.2.1 CTC Identification

CTCs have an extremely low frequency in blood circulation; the scarcity of CTCs and the abundance of leukocytes amongst which they are present demand extreme sensitivity and specificity from their detection methods. Additionally, tumours, and with them probably also CTCs, are heterogeneous within and between patients (Allard et al., 2005; Navin et al., 2011; Sieuwerts et al., 2009). It is this heterogeneity, caused by the existence of CTC subpopulations with distinct biological roles or simply reflecting the heterogeneity that is also present in solid tumours, that presents an obstacle in the development of an assay that would be able to detect all

types of CTCs in every patient. Moreover, because most assays rely on the expression of epithelial markers on CTCs, cells that have undergone epithelial-to-mesenchymal transition (EMT) upon entering the bloodstream could remain undetected. The mesenchymal phenotype of CTCs that undergo EMT promotes motility. Classic EMT properties induce aberrant cellular motility while classic stemness properties, including apoptosis resistance, transient quiescence, and self-renewal capacities, allow for survival during dissemination, colonization at the metastatic site, eventual drug resistance and the long-term maintenance of CSCs (Scatena, Bottoni, & Giardina, 2013).

Indeed, cancer cells must undergo a reverse mesenchymal-to-epithelial transition (MET) to acquire the ability to proliferate and thus form a metastatic tumour (Joosse, Gorges, & Pantel, 2014) (

Figure 1.2). The detection of CTCs lacking epithelial markers may, however, be of crucial clinical importance as EMT is thought to be mandatory for haematogenous metastasis and is associated with poor prognosis (Micalizzi, Farabaugh, & Ford, 2010; P. Savagner, 2010; Pierre Savagner, 2015; Willipinski-Stapelfeldt et al., 2005). It is also hypothesised that migratory cancer stem cells may be the source of distal tumour metastases in some cancers. For a tumour cell to produce a metastatic lesion, it must first detach from its niche of origin and survive outside, invade, and navigate through neighbouring tissues, and re-associate and proliferate in a supportive microenvironment. During EMT, the epithelial features of a cell, such as the expression of the attachment molecule E-cadherin, are suppressed to gain a more motile phenotype (Thiery, Acloque, Huang, & Nieto, 2009). In addition, tumour cells undergoing EMT have been found to be enriched in cancer stem cell-associated features, suggesting that the tumour cell plasticity facilitates the EMT and MET processes during metastasis (Brabletz, Jung, Spaderna, Hlubek, & Kirchner, 2005; T. Chen, You, Jiang, & Wang, 2017).

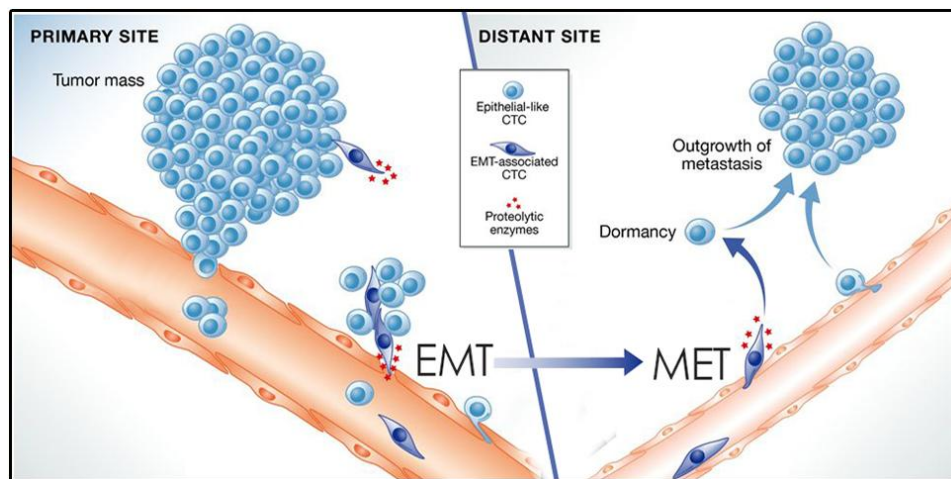


Figure 1.2: Dissemination of tumour cells. Tumour cells may enter the EMT. Once at a distant site, tumour cells may extravasate, undergo MET, and grow locally to become a metastasis or remain in dormancy (Joosse et al., 2014).

CTC technologies have evolved rapidly to obtain the highest and most relevant CTC count: flow cytometry-based methods (Pachmann et al., 2005; Scholtens et al., 2011), microfluidic chips, and quantitative reverse transcriptase PCR (qRT-PCR) (Bosma et al., 2002), yet there are none that have FDA approval other than CellSearch.

Any ideal CTC technology should offer high throughput, a minimal handling of the blood sample (sampling whole blood) that can separate live CTCs with high sensitivity and specificity. Immunoaffinity-based technologies offer both sensitivity and specificity, albeit with dependence on the known biomarkers. A high-throughput system that exploits CTCs' physical properties, requires minimal pre-manipulation of whole blood, and can operate with either positive selection or negative depletion approach seems to be most promising for lung cancer CTC isolation. Furthermore, the efficiency of positive selection future depends on the discovery of new lung cancer specific surface markers such that a cocktail of specific capturing antibodies can be applied to target a broader range of lung CTCs (Yu Ling, Ng Rui Shu, Xu Yang, Dong Hua, Wang Yingjun, 2013).

1.2.1.1 Immunoaffinity based isolation

There are two main CTC immunoaffinity isolation methods. One is anti-epithelial cell adhesion molecule (EpCAM) dependent while the other is EpCAM independent (Zhang J, Chen K, 2016). EpCAM is widely employed as the target in almost all immunoaffinity-based CTC isolation strategies. The FDA approved CellSearch technology utilizes EpCAM-coated magnetic beads to isolate CTCs in a multitude of cancers in spite of limited detection efficiency when used alone (it detects only 33 % of CTCs in lung cancer) (Bellail, Olson, & Hao, 2014; Gorges et al., 2016; Krebs et al., 2011; P. Went et al., 2006; P. T. Went et al., 2004; Willipinski-Stapelfeldt et al., 2005). When immunomagnetic principles are utilised in combination with microfluidic chips coated with EpCAM, the efficiency of lung cancer capture can rise to 100 % (Nagrath et al., 2007; Stott et al., 2010; Yoon et al., 2013; Zhang J, Chen K, 2016). Their advantages are high sensitivity, low white blood cell contamination, and preserving the viability of CTCs. The drawbacks are low flow rates (1-3 ml/hour) and a requirement for antibody-antigen interaction. Another problem with EpCAM dependent methods is that they can only capture a subset of CTCs and miss cells undergoing the EMT, which may carry important molecular information about primary tumours. Therefore developing enrichment technologies that can effectively capture CTCs of varying phenotypes remains a complex, challenging, and constantly evolving endeavour (Andree, van Dalum, & Terstappen, 2016; Ferreira, Ramani, & Jeffrey, 2016; W. L. Hwang, Pleskow, & Miyamoto, 2018).

1.2.1.2 Size based isolation

CTCs are often larger in size and may have a different specific gravity than blood cells. Therefore label-free approaches for isolation of CTCs rely on physical properties such as size, deformability, dielectric susceptibility, and negative selection of White Blood Cells (WBCs) (De Giorgi et al., 2010; Desitter et al., 2011; Fan et al., 2015; Hosokawa et al., 2013; T. Huang et al., 2014; Hyun, Kwon, Han, Kim, & Jung, 2013; M. S. Kim et al., 2012; Lin et al., 2010; M. Da Zhou et al., 2014). Therefore they can be separated from blood cells either by physical filtration or by hydrodynamic forces (Hou et al., 2011). The advantage of this approach is that they operate at relatively high flow rates and are therefore able to enrich rare CTCs from blood samples. The isolated cells' viability is not compromised and therefore they can be collected for further molecular characterizations, single cell analysis, and culturing. The limitations of physical-based techniques are related to heterogeneity of tumour cells (CTCs within a patient can range in size and may overlap with the size of blood cells) and contamination with blood cells resulting in lower specificity compared with antigen-based systems (Harouakaa, Kangb, Zhenga, & Cao, 2013).

The ISET (isolation by size of epithelial tumour cells) technology, isolating CTCs based on their larger size, is among the earliest EpCAM independent approaches which filter CTCs from blood cells as they pass through a membrane filter (Vona et al., 2000). CTCs were detected in 80 % of samples from stages IIIA to IV NSCLC patients using ISET compared with 23 % using Cell Search (Krebs et al., 2012). Using the same approach, recent reports show that CTCs were present in 65 % of NSCLC patients (Mascalchi et al., 2016).

1.2.2 CTCs as biomarkers for early lung cancer detection

More sensitive detection of CTCs provides new opportunities for early diagnosis of lung cancer since CTCs can be shed by the primary tumour even at early stages of tumour development (Rhim et al., 2012). The invasive character of lung cancer could permit its early diagnosis through the sensitive and diagnostic detection of CTCs as they can be detected in patients without clinically detectable lung cancer, thus lowering the possibility of recurrence after surgery (Ilie et al., 2014).

CTCs are valuable for diagnosis, monitoring therapy and prognosis in primary lung cancer. As a reproducible indicator of disease status that may be superior to imaging when using CD45-FISH method with increasing sensitivity by including cells deficient in epithelial markers. CTCs were isolated from lung cancer patients of various stages. More than half of patients were in an early stage of lung cancer (X. Chen et al., 2015; Y. Y. Chen & Xu, 2014; Lou et al., 2013). Their suitability for prediction of malignancy of the lung lesions as a way to circumvent sampling bias by solid biopsy has been also shown (Fiorelli et al., 2015). For a malignant circulating cell count of greater than 25, sensitivity and specificity were 89 % and 100 %, respectively. The count was significantly correlated with stage, size, and standard uptake value of the primary tumour. In 72 % of cases, the malignant circulating cells allowed a specific histologic diagnosis of the corresponding primary tumour after immunohistochemical analysis (Fiorelli et al., 2015; Z. Zhang et al., 2014). While during lung cancer progression some tumour cells acquire new characteristics – increased expression of mesenchymal markers (N-Cadherin, $\alpha 5\beta 1$ Integrin,

$\alpha\text{v}\beta 6$ Integrin, Vimentin, Type I Collagen, Laminin 5) and loss of epithelial markers (E-Cadherin, Claudins, Occludins, Desmoplakin, Type IV Collagen, Laminin 1) – it is crucial to consider those changes when trying to detect CTCs successfully (Figure 1.3) (Shih & Yang, 2011).

Low abundance of CTCs still presents a challenge for their detection in early stages of lung cancer and that calls for a compiling development of more sensitive and specific technologies such that more inclusive characterisation methodologies will aid in early detection of lung cancer.

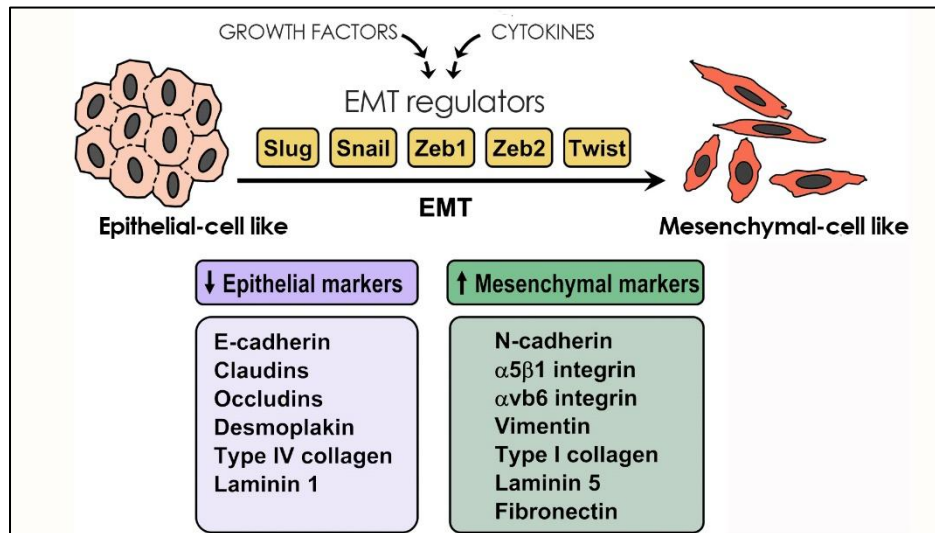


Figure 1.3: An overview of epithelial and mesenchymal markers changes during EMT. Under the effect of growth factors and cytokines, the EMT regulators transform the cancer cells from epithelial-cell like to mesenchymal-cell like with suppression of epithelial markers and expression of mesenchymal markers (Shih & Yang, 2011).

1.3 Cancer Stem Cells

1.3.1 Normal Stem Cells

Normal adult stem cells are defined as cells that have the ability to maintain tissue homeostasis by replacing cells lost through damage and injury. The pluripotent embryonic stem cells derived from the embryonic blastocyst stage can generate mature cells and tissues of the three primary germ layers. Only the stem cell lineages comprising the tissue are generated for a specific tissue type. These adult stem cells usually represent a small percentage of the tissue mass (Tannishtha, Morrison, Clarke, & Weissman, 2001).

To achieve their functional role, stem cells should possess two main characteristics - self-renewal and differentiation. Self-renewal refers to the ability of the cells to maintain a hierarchy of daughter cells in the stem cell pool, with progenitor cells forming the early generations. Progenitor cells are not identical to the parental stem cells but they retain many of their characteristics initially and are capable of cell proliferation. Besides normal tissue turnover and maintenance, their ability to self-renew also enables the expansion of the stem cell compartment in response to intrinsic and/or extrinsic stimulation. This triggers massive proliferation of a tissue-specific undifferentiated cell pool in the organ or tissue and gives rise to a larger bulk of non-stem cells that have a definite life span. With each subsequent cell division, progenitor cells lose their self-renewal potential as they differentiate to generate more mature cells (Lobo, Shimono, Qian, & Clarke, 2007; Spillane & Henderson, 2007).

1.3.2 Cancer Stem Cell Models

It has been known that tumours from patients exhibit significant heterogeneity with respect to cell morphology, surface marker, cell proliferation potential, and response to therapy. One explanation proposes that all tumour cells are biologically equivalent, but heterogeneity is due to combinations of stimulations from extrinsic and intrinsic sources that result in random responses. This explanation stands for the stochastic model of cancer development. Another possible explanation of cancer cell heterogeneity is the central idea of the cancer stem cell model. It proposes that, comparable with the normal tissue homeostasis, there is a hierarchical organization in tumours during their development and maintenance. For normal tissues, the normal stem cells reside at the apex of the hierarchy, while for tumours, this is valid for the cancer stem cells (Dick, 2008). The stochastic and cancer stem cell models need not be mutually exclusive because, theoretically, a new clone of cancer stem cells might also be generated stochastically by intrinsic or extrinsic carcinogenic factors. The essential difference between these two models is that in the stochastic model, cancer stem cells arise randomly and every tumour cell has the potential to give rise to progeny cells (although the chance for generating novel stem cell clones is low because this would require the mutagenic stimulus to affect specifically genes which are crucial for acquiring stem cell properties). For the cancer stem cell model, there is only one distinct subpopulation of cells that plays the role of cancer stem cells essential for sustaining the cancer (Dick, 2009).

The stochastic model suggests every tumour cell is biologically homogeneous. The behaviour of the tumour cell is determined by intrinsic stimuli (e.g. transcription factors and hormones) or extrinsic (e.g. microenvironment and mutagens). Those stimuli would randomly (stochastically) affect the tumour cells. Because of this, every tumour cell might form its own clone within the tumour, resulting in cell heterogeneity (Dick, 2008).

On the other hand, the cancer stem cell model hypothesises the development and maintenance of a tumour resembles those of normal tissues where cells are arranged in a hierarchy in which the stem cell is at the apex of this system. Cancer stem cell is the one that has acquired sufficient oncogenic mutations for transformation and is therefore capable of developing a cancer. The tumour cell of origin, also referred as tumour initiating cell is the progenitor cell capable of self-renewal and differentiation. According to this model, a cancer stem cell is biologically distinct from the rest of the bulk tumour mass (Dick, 2009). Just as normal stem cells, most cancer stem cells are highly clonogenic, and compared to non-cancer stem cells have a greater capacity for long term proliferation. These two main characteristics make them the principle agents in tumour cell heterogeneity and tumour progression (Clarke et al., 2006; Clarke & Fuller, 2006; Tannishtha et al., 2001; Wicha, Liu, & Dontu, 2006) (Figure 1.4). The epithelial to mesenchymal transition (EMT), which is a reversible process and can transiently induce stem-like cells phenotypes, give an idea of attainable expansion of static the CSC model to the fluid CSC model also termed the CSC plasticity model. This model proposes CSC plasticity- a variable CSC population may evolve from a variety of cell types within growing tumor mass by subsequent mutations or epigenetic modifications. Various CSC sub clones may give a rise to more aggressive secondary CSCs as a result of evolutionary pressure of therapies (E. S. Molina, Pillat, Moura-Neto, Lah, & Ulrich, 2014).

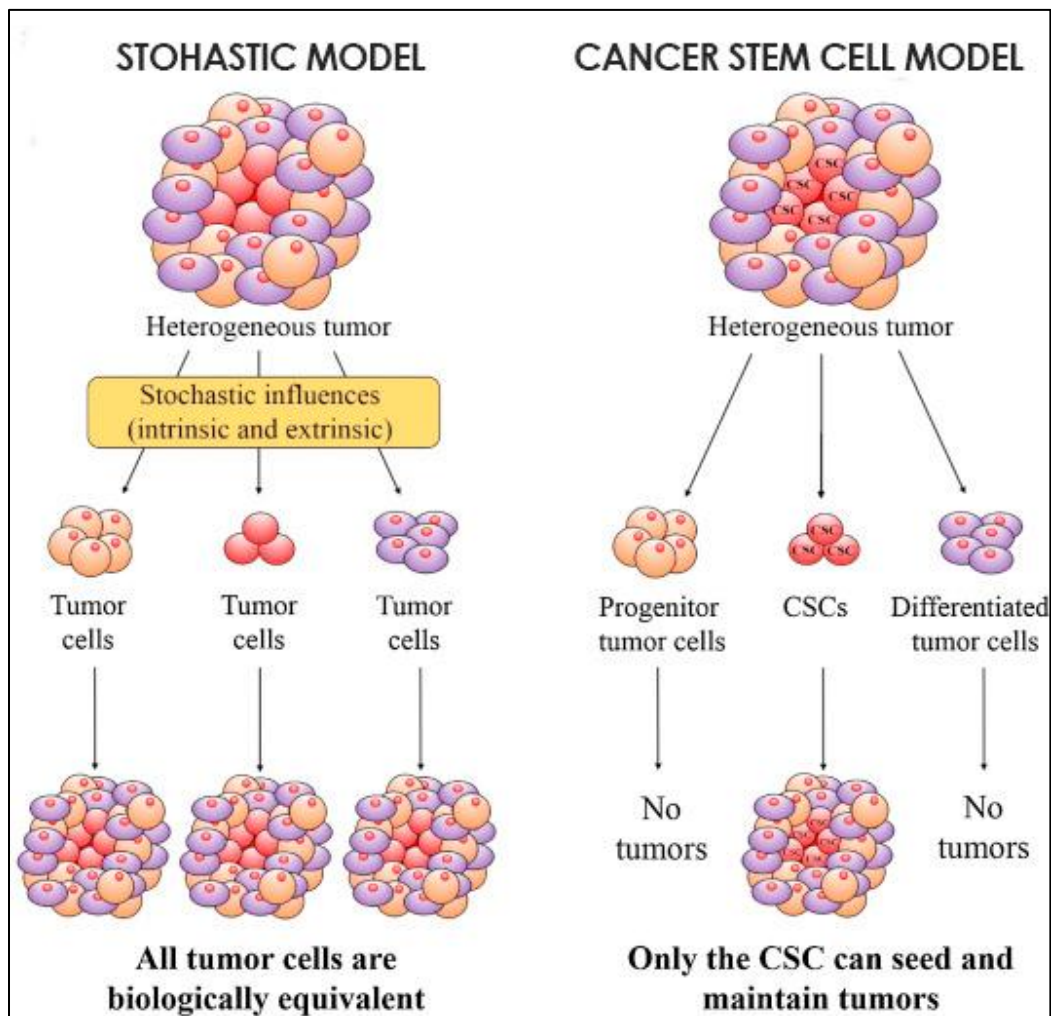


Figure 1.4: Models of tumour growth. In the stochastic model of cancer, tumour mass consists of biologically equivalent cells, those are under intrinsic and extrinsic influences capable of forming a new tumour. The cancer stem cell model states that only CSCs inside tumour's heterogeneous mass have capacity to initiate and maintain a new tumour (adopted from Koren & Fuchs, 2016).

1.3.3 Lung Cancer Stem Cell Markers

The presence of a stem-like clonogenic subpopulation in human lung cancer was demonstrated more than thirty years ago, when it was observed that only a very small proportion of SCLC and lung adenocarcinoma cells from patient samples could generate colonies in soft agar. Colonies inoculated into nude mice were able to generate SCLCs and lung adenocarcinomas similar to their primary lesions. That led to the idea that these rare but clonogenic cell were enriched with lung cancer stem cells (Carney, Gazdar, & Minna, 1980).

In 2007, Ho et al showed that with the use a Side Population (SP) assay human lung cancer stem cells could be identified and selected (Ho, Ng, Lam, & Hung, 2007). The Side Population assay, first described by Goodell and colleagues to select for human hematopoietic stem cells (Goodell, Brose, Paradis, Conner, & Mulligan, 1996), relies on the ability of ABC transporters expressed in stem cell populations (S. Zhou et al., 2001), to efflux the fluorescent Hoechst 33342 dye. Hoechst 33342 dye excluding cells named Side Population cells (SP cells) have been found to have enriched stem-like properties (Hirschmann-Jax et al., 2004). Also from human lung cancer cell lines isolated SP cells were able to raise a xenograft in mice, while non-SP cell were not showing that ability (Ho et al., 2007). One of the major concerns about using the SP assay is that

the Hoechst 33342 dye is actually a DNA binding dye that has cytotoxic effects to cells. The decrease in the ability to form clones and tumours *in vitro* and *in vivo* by the non-SP cells could be due to artefacts caused by dye cytotoxicity. The non-unified assay's performance (differences in incubation time, dye concentration, cell concentrations, and gating variability) also often lead to variable SP phenotypes between the experiments (Montanaro et al., 2004; Zhong et al., 2007).

Another strategy for isolating human lung cancer stem cells is using surface stem cell markers that discriminate diversity of intra-tumoural cell heterogeneity. This strategy has been proven successful in several solid tumour types where cancer stem cells can be identified and isolated using fluorescence-activated cell sorting (FACS) (Visvader & Lindeman, 2008).

In 2008, Eramo and colleagues identified rare, highly tumorigenic, self-renewing CD133+ cells in both NSCLC and SCLC specimens (Eramo et al., 2008). CD133 (also called PROM1) is a cell surface glycoprotein containing five trans-membrane domains and two glycosylated extracellular loops (Mizrak, Brittan, & Alison, 2008). CD133 is a marker of the human hematopoietic stem cells (Yin et al., 1997) and neural stem cells (Uchida et al., 2000). Furthermore it is also reported to be a marker for selecting brain (Singh et al., 2004), colon (Dalerba et al., 2007; O'Brien, Pollett, Gallinger, & Dick, 2007; Ricci-Vitiani et al., 2007) and pancreatic (Li et al., 2007) cancer stem cells. For lung cancers, a rare cell subpopulation expressing CD133 in both NSCLC and SCLC specimens were discovered which were highly tumorigenic and self-renewing. CD133+ cells isolated from the tumours were able to form cell spheres that were resistant to chemotherapy. But unlike other well-established cancer stem cell markers, CD133 expression level was not associated with patient prognosis (Salnikov et al., 2010; Tirino et al., 2009). CD133+ cells were not detected in many lung cancer samples, suggesting that cells with other phenotypes characteristics are also required for self-renewal and cancer initiation in these cases (Bertolini et al., 2009; Salnikov et al., 2010; Tirino et al., 2009). Meng and colleagues have shown that both CD133+ and CD133- subpopulations of the lung cancer and some cell lines contain similar number of cancer stem cells. That suggest that CD133 alone cannot be used as a stem cell marker and that new markers with higher specificity and accuracy in identifying lung CSCs should be further explored (Meng, Li, Wang, Wang, & Ma, 2009).

The discovery that CD90 positive cells display higher tumorigenic capacity suggests that CD90 could be another valuable lung cancer stem cell marker (Yan et al., 2013). CD90 (Thy-1) is heavily glycosylated. The glycoposphatidylinositol (GPI)-anchored cell surface protein expressed mainly in leukocytes affects numerous non-immunologic biological processes, including cellular adhesion, neurite outgrowth, tumour growth, migration, and cell death (Rege & Hagood, 2006). CD90 expression was identified in murine breast CSCs (Cho et al., 2008), primary high-grade glioma CSCs (He et al., 2012), hematopoietic stem cells (Baum, Weissman, Tsukamoto, Buckle, & Peault, 1992), and bone marrow-derived mesenchymal stem cells (Dennis et al., 2007) and in liver malignancy (Conigliaro et al., 2015; Yang et al., 2008).

Further studies of lung cancer revealed the possibility that CD44 is a marker of lung cancer stem cells. CD44 is a membrane-bound glycoprotein and has been reported as a cancer stem cell marker in many cancer types: breast cancer (De Beca et al., 2013), colon cancer (Botchkina et al., 2009) and liver cancer (Zhu et al., 2010). CD44+ cells showed significantly a higher expression of stem cell genes and its expression was found to correlate with a poor prognosis in squamous cell carcinoma (Leung et al., 2010).

Stem cell population can also be selected and isolated by using a method based on aldehyde dehydrogenase 1 activity (ALDH1). It belongs to a family of cytosolic enzymes and it may play a vital role in the formation of retinoic, which is a potent modulator for gene expression and tissue differentiation (Yoshida, Rzhetsky, Hsu, & Chang, 1998). High ALDH1 activities have been proposed in hematopoietic stem cells for preserving the undifferentiated cellular state (Murimoto et al., 2006). High ALDH1 activity has also been used to isolate cancer stem cells from human brain (Rasper et al., 2010), breast (Morimoto et al., 2009), colon (Fitzgerald, Rangan, Dobbs, Starr, & Sigounas, 2014), head, and neck cancers (Y.-C. Chen et al., 2009). In the lung, expression of ALDH1 was found to be elevated in lung stem cell niches during malignant transformation (Patel et al., 2008). ALDH1 expression positively correlates with the stage and grade of lung tumours and correlates with a poor prognosis for the patients with early-stage lung cancer (F. Jiang et al., 2014). Recent studies support that ALDH1 expression and its significance likely differ with respect to environmental insult, location within the lung, and pathophysiological type, which implies that ALDH expression and activity would not be useful as a single marker for lung cancer stem cells (Templeton, Miyamoto, Babu, Munshi, & Ramesh, 2014).

As different studies revealed novel lung cancer stem cell markers and identified those cells using different approaches (side population phenotypes, cell surface markers, enzyme ALDH1 activity) their work was disapproved by the others. Therefore, until now there is no truly reliable way to identify lung cancer stem cells, which leaves that field of research still broadly open.

1.4 Aptamers

In the early 1990s, several research laboratories were independently experimenting with what Szostak's group termed "*in vitro* selection" (Ellington & Szostak, 1990; Lauthon & Szostak, 1995; Schneider, Feigon, Hostomsky, & Gold, 1995; Tuerk & Gold, 1990). To describe molecular recognition properties of those short single stranded DNA or RNA oligonucleotides, they coined the term 'aptamer' using the Latin word "*aptus*", meaning fitting and the Greek word "*meros*", meaning "particle". But naming aptamers was not nearly as interesting as discovering that their highly defined three-dimensional structure helps them to bind to their targeted molecules with nanomolar affinities and high specificity and that they can compete in many other properties quite well with antibodies (Cerchia, Giangrande, Mcnamara, Franciscis, & Pansini, 2009; Kong & Byun, 2013) (Figure 1.5)

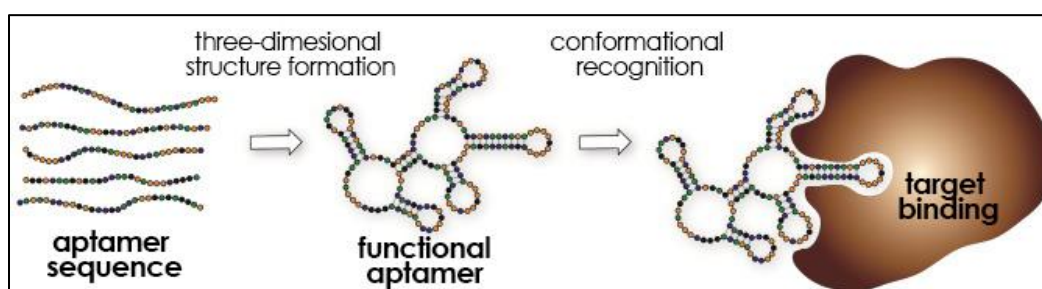


Figure 1.5: Aptamer conformational changes and recognition of targets to form an aptamer-target complex (adopted from Darmostuk, Rimpelová, Gbelcová, & Ruml, 2015).

The special quality of aptamers is their small size, low immunogenicity, and low molecular weight (5-25 kDa), which correspond to their high binding affinity to targets. Similar to conformational recognition that mediates antibody-antigen recognition and complex formation, aptamers bind to their cognate targets using high-specificity and -affinity through van der Waals forces, hydrogen bonding, electrostatic interactions, stacking of flat moieties, and shape complementarity (Hermann & Patel, 2013) with dissociation constants (K_d) usually ranging from pico- to nanomolar (Nimjee, Rusconi, & Sullenger, 2005). Aptamers bind to a wide range of targets from metal ions (Kawakami, Imanaka, Yokota, & Sugimoto, 2000), small organic compounds (Mann, Reinemann, Stoltenburg, & Strehlitz, 2005; Mannironi, Di Nardo, Fruscoloni, & Tocchini-Valentini, 1997), biological cofactors (Holland, Henry, Whinna, & Church, 2000; Lauthon & Szostak, 1995), metabolites (Bruno, Carrillo, Phillips, Vail, & Hanson, 2008), proteins (Bock, Griffin, Latham, Vermaas, & Toole, 1992; Ruckman et al., 1998; White et al., 2001), and whole organisms such as a virus (Dalerba et al., 2007; Tang, Parekh, Turner, Moyer, & Tan, 2009), bacteria (Hamula, Le, & Li, 2011; Y. S. Kim, Chung, Song, Jurng, & Kim, 2014), yeast (Kolesnikova et al., 2010; Thomas et al., 1997), parasites (Ulrich, Magdesian, Alves, & Colli, 2002) or mammalian cell (Daniels, Chen, Hicke, Swiderek, & Gold, 2003).

To improve aptamer bioavailability, strategies have been developed to chemically modify backbone or side chains, incorporate unnatural nucleotides, and cap aptamer ends (Gao, Zheng, Jiao, & Wang, 2016; Sun, Tan, & Zu, 2016). These advances, along with the parallel progresses in the nucleic acids chemistry and cellular delivery fields, have allowed for the rise of a new hope in developing aptamers as efficient molecular tools for diagnostics and therapeutics (Romero-l & Berzal-herranz, 2017). Several post-selection optimizations strategies improves specific properties of aptamers and thus the selected type of improvement can be adjusted directly to the application purpose (Gao et al., 2016). So far, the applications for aptamers are growing rapidly and they have covered many aspects including the development of methods to detect or monitor disease, to screen out a specific aptamer against key molecules involved in pathological or virus infectious process, and to map the cell surface coat of stem cells (K. Liu, Lin, & Lan, 2013).

1.4.1 Generation of Aptamers

There is a high zeal for development of molecules with high-specificity and -affinity towards selected targets. There are two main approaches to design ligands that can bind with high-affinity

and high-specificity molecules - rational design and combinatorial chemistry. Rational design relies on chemical wisdom, intuition, and, to some degree, luck to identify molecules capable of interacting with the target. In combinatorial chemistry, enormous numbers of molecules are produced to form libraries and these molecules are tested to find potential candidates to be ligand (Bowser, 2005). Aptamers are produced and selected with the use of combinatorial chemistry. In 1990 a different combinatorial technique named *in vitro* selection (Ellington & Szostak, 1990), *in vitro* evolution (Joyce, 1989), or SELEX (Systematic Evolution of Ligands by EXponential enrichment) (Tuerk & Gold, 1990) were introduced for the production of aptamers.

The conventional SELEX method mainly consists of the following three steps: selection, partitioning, and amplification (Figure 1.6). Before the selection, a library of oligonucleotides is synthesized, which generally contains up to 10^{15} different unique sequences (Tuerk & Gold, 1990). Each unique sequence contains 20-50 nucleotides length random region flanked by two conserved primer binding sites, which are used for PCR amplification by annealing primers. In the selection step, the library is incubated with target molecules for the designated time. After incubation, the unbound and bound sequences are separated. The target-bound sequences are amplified by PCR (for DNA aptamers) or reverse transcription PCR (RNA aptamers). The newly obtained sub-pool is further utilized for the next selection round (Zhuo et al., 2017). With each of the following rounds the stringency of selection conditions (use of larger starting library sequence diversity, gel purification of the subsequent pools, introduction of counter-selection) is increased. After several selection rounds (~20), the enriched sequences are cloned, sequenced, analysed for structural similarities and their binding abilities are further evaluated (Hamula et al., 2015). Generally, it takes weeks to months to obtain specific aptamer candidates, and the hit rates are low. Thus, the obtainment of high quality aptamers against relevant targets still remains a bottleneck. Once knowledge of the particular aptamer sequence is elucidated, production can be easy and rapid via enzymatic *in vitro* transcription or chemical synthesis. Novel modifications made the selection process more efficient, cost-effective, and significantly less time-consuming (Darmostuk et al., 2015).

SELEX accelerated the generation of aptamers with a capacity of recognizing virtually any class of target molecules with high affinity and specificity. Since then conventional SELEX and its derivatives have been designed as the targets to generate aptamers conveniently and easily (Cerchia, Esposito, Jacobs, Tavitian, & de Franciscis, 2009; Chandra & Gopinath, 2007; Ellington & Szostak, 1990; Jayasena, 1999; Shamah, Healy, & Cload, 2008; Stoltenburg, Reinemann, & Strehlitz, 2007). In the last decade technological developments and emerging concepts in SELEX and aptamer fields are emerging, which have gained significant momentum by incorporating and adapting various other technologies such as microarrays, next-generation sequencing, and microfluidics (Ozer, Pagano, & Lis, 2014).

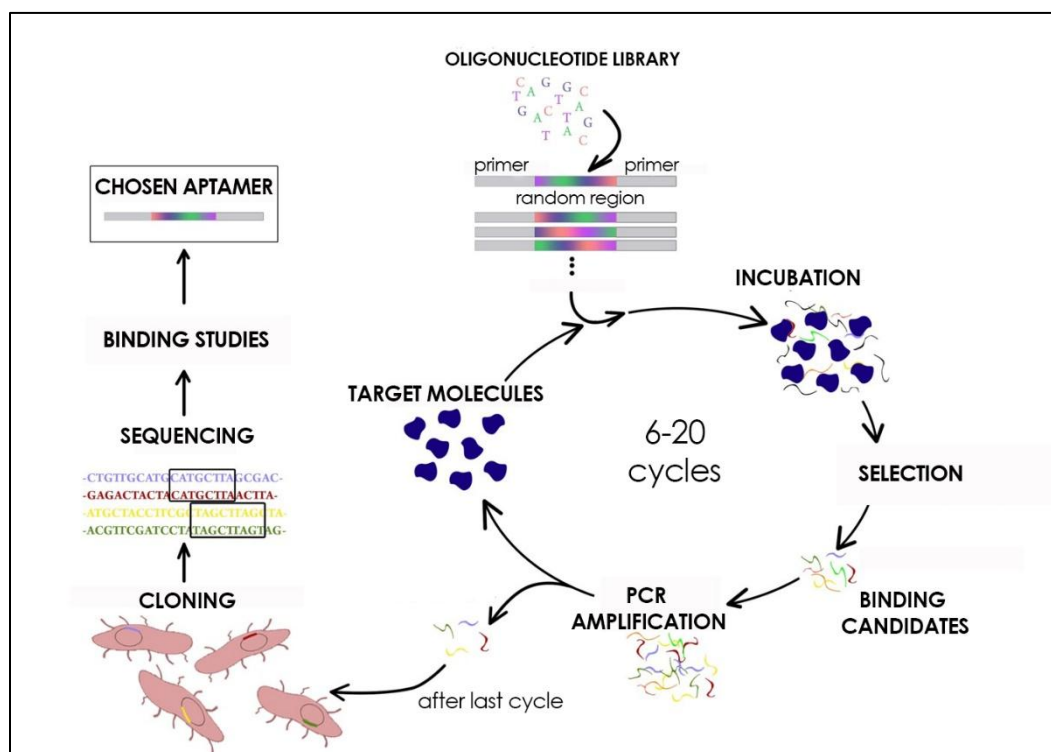


Figure 1.6: SELEX (Systematic Evolution of Ligands by EXponential enrichment) workflow overview.

1.4.1.1 Cell-SELEX

Cell-SELEX does not require any prior knowledge of the protein conformation and their purification by processes that may disrupt the native conformation. Cell-surface, membrane-bound molecules remain in their native environment and therefore retain native folding structure with possible post-translational modifications throughout the selection process (Ye et al., 2012). It is a particularly promising selection strategy for various applications, including cancer research and therapy. Useful probes can be developed to potentially differentiate tumour cells from normal cells, as well as to differentiate between two different types of cancers. Live cells of different cancers have been used with this approach and as a result, a significant number of aptamers have been generated (Sefah, Shangguan, Xiong, Donoghue, & Tan, 2010). Not only against cancer cells, Cell-SELEX is also a powerful tool for selecting aptamers for disease specific biomarker discovery by aptamers. As such, Ulrich et al. (2002) pioneered on selection of nuclease-resistant RNA aptamers binding with high affinity and specificity to receptors of host cell matrix molecules on protozoan *Trypanosoma cruzi* causing Chagas' disease. After eight consecutive selection rounds aptamers sharing consensus motifs were active in inhibiting invasion of monkey kidney cells by *T. cruzi* *in vitro*. The development of aptamers using Cell-SELEX approach also provides a powerful tool for detection of secreted proteins of infectious agents such as the malaria parasite with the most severe form caused by *Plasmodium falciparum*. The Cell-SELEX technique is a suitable instrument for detection and identification of *P. falciparum* proteins in the plasma membrane of infected erythrocytes (Ulrich & Wrenger, 2009).

The basic partitioning technique involves gentle centrifugation for cells in suspension or washing for adherent cells. Another common approach to separate bound from unbound oligonucleotide sequences is fluorescence activated cell sorting (FACS), which is also used to characterise the affinity and specificity of the selected aptamers (Kim et al., 2014). Typically, Cell-SELEX consists of two different sets of selection: a positive selection against target cells and a negative or counter selection against non-target cells. Positive selection commonly occurs against tumour cells, resulting in aptamers that are capable of distinguishing between cancerous and non-cancerous tissue as well as other cell combinations have been used to select more specific aptamers such as those that distinguish between mature and immature dendritic cells (Berezovski, Lechmann, Musheev, & Mak, 2008).

Selection conditions vary depending on the intended future application of the aptamer. As the majority of aptamers are developing further *in vivo* applications, they are selected under physiological conditions (37 °C and pH of 7-8) as later change in conditions lower their affinity and make them non-functional and unusable for *in vivo* testing (Hicke et al., 1996). The bound sequences are then eluted from the target cell by heat dissociation and amplified via PCR. When the negative round is introduced, the unbound sequences that are collected and used for the subsequent round of positive selection. Negative selection reduces the amount of non-specific sequences collected during positive selection and ensures that the aptamers that are selected are unique for the desired target. The progress of selection during Cell-SELEX is typically monitored via flow cytometry using relative fluorescence units (RFUs) representing the number of events. With the progression of the selection the number of RFUs increase indicating success in selection rounds. Selection typically continues until no significant increase is seen in RFUs from one round to the next (Sefah et al., 2010).

1.4.1.1.1 Non-specific binding Non-specific sequences are also collected during selection due to dead cells taking up nucleic acids from the Cell-SELEX suspension. Several studies have shown that dead cells become increasingly permeable to both RNA and ssDNA sequences and take up a large amount of sequences non-specifically (Avci-adali, Metzger, Perle, Ziemer, & Wendel, 2010). The number of sequences collected from dead cells is exceedingly greater than the number of sequences that typically adhere to a living cell's membrane (Raddatz et al., 2008). Further amplification, decreased efficiency and loss of potential best binders from the pool must be therefore avoided. Presence of dead cells is therefore evaluated with propidium iodide (PI) stain coupled with flow cytometry (Jones & Senft, 1985; Sefah et al., 2010). For further separation and collection of living and dead cells based on their light scattering characteristics a fluorescence-activated cell sorting (FACS) can be used (Raddatz et al., 2008).

1.4.1.1.2 PCR amplification After incubation and elution, obtained sequences need to be amplified and prepared for the following round. Amplification with PCR reaction is important for maintaining the specificity and reduction of potential non-specific by-products. The accurate annealing of primer regions to desired template controls reaction's specificity (Musheev & Krylov, 2006). For the majority of cell SELEX procedures the annealing temperature is set to just above the melting temperature (T_m) of the primer, that is based on the length and GC content of the sequence (Rychlik, Spencer, & Rhoads, 1990). Unwanted by-products may be part of genomic DNA that has been released by target cells and may be collected along with potential aptamers (Pan & Clawson, 2009). Unwanted by-products can also be originating from non-specific amplification of library. The input template concentration is therefore an important factor that limits the specificity of a PCR reaction. The amplification of target sequences typically plateaus in the nM range after which unwanted by-products begin to form. Over-amplification of target sequences results in their degradation into smaller by-products (Nagy et al., 2005). In order to ensure that all potential aptamer sequences collected in the initial SELEX rounds are accurately amplified and remain in the selection pool the number the PCR protocol has to be outlined precisely.

1.4.2 Aptamers versus Antibodies

Aptamers can provide an excellent alternative to antibodies due to their similar recognition properties (Table 1.1). As chemical antibodies, aptamers are superior in their smaller size (Figure 1.7), higher stability (dehydrated form) lack of immunogenicity, adaptive modifications, easy chemical synthesis, and low cost cell-free production (A. Chen & Yang, 2015). The production does not require any animals or specific cell lines, thus they can be raised against toxic molecules or non-immunogenic targets. Raising antibodies is dependent on living organisms and that makes them sensitive to their environment. Aptamers are more resistant to pH and temperature changes and the selection condition can be modified according to the final usage of the aptamer (Rozenblum, Lopez, & Vitullo, 2016). Experiments testing accuracy and specificity of aptamers and antibodies have proven that aptamers show more accurate and specific results compared to antibodies. Aptamers have also been used in ELISA-like assays and flow cytometry (Blank, Weinschenk, Priemer, & Schluesener, 2001; Zeng, Zhang, Zhao, Sheehan, & Tung, 2010). To date, antibody-based assays have also been developed for *in vivo* applications but, in most cases, antibodies have failed to reach expected adequate sensitivity because of the toxicity profile *in*

vivo. Aptamers display low to no immunogenicity when administered in preclinical doses 1000-fold greater than doses used in animal and human therapeutic applications (Rozenblum et al., 2016). Their small size and polyanionic nature, helps them to accumulate at the tumour site and show rapid blood clearance, minimizing their residence in liver and kidney. In addition, aptamers include faster excretion than antibodies due to the small size and susceptibility to serum degradation when unmodified aptamers are used, but by modifying their structure and adding a stabilising group, it helps to resolve that problem. Clinical studies have confirmed that aptamers are thermally stable, have an unlimited shelf life, non-toxic and non-immunogenic molecules, and so their applications have far exceeded their limitations (Blank et al., 2001; K. Y. Lee et al., 2010; Proske, Blank, & Buhmann, 2005).

Aptamers have an unleashed potential to bypass the antibody's limitations. The aptamer research is already in high-pace development and, with automation and further improvements of selection methods, a high-throughput aptamer identification seems entirely possible. The value of aptamers is prominent in applications where antibodies are inadequate.

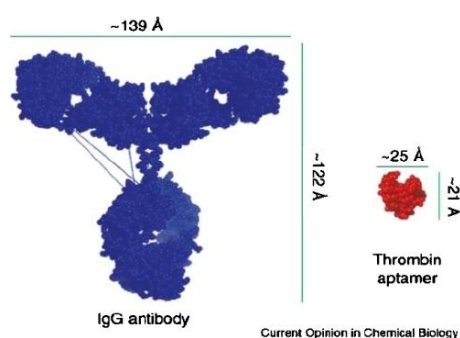


Figure 1.7: Size comparison of an aptamer with an antibody (IgG immunoglobulin G) (adopted from J. Lee, So, & Jeon, 2008).

Table 1.1: Comparison of Aptamers with Antibodies (Blind & Blank, 2015; A. Chen & Yang, 2015).

	APTAMERS	ANTIBODIES
TARGET RANGE	Almost target from ions to whole cells, including non-immunogenic or toxic targets	Limited to non-toxic and non-immunogenic targets
DEVELOPMENT TIME	Several weeks	Several months
SELECTION	<i>In vitro</i> selection under variety of conditions	Selection limited to physiologic conditions by animal immunization
PRODUCTION	Low cost chemical synthesis	Expensive and time consuming
BATCH ACTIVITY	Consistent	Variable
SIZE	Small (8-12kDa)	Large (150 kDa)
HANDLING & STORAGE	Stable at room temperatures	Required continuous cold chain
STABILITY	Tolerant to pH and temperature Redox-insensitive Difficult to aggregate Can withstand repeated rounds of denaturation without loss of function	Sensitive to pH and temperature Redox-sensitive Easy to aggregate Easy to denature
SHELF LIFE	Long (several years)	Limited (~6 months)
<i>IN VIVO</i> USAGE	Low immunogenicity High bioavailability	High immunogenicity Low bioavailability
DOWNSTREAM APPLICATIONS	Functional under broad range of conditions Selected conditions can base on intended end use	Only work close to physiological conditions
AFFINITY MODIFICATIONS	Low nanomolar to picomolar Easy to modify with low costs	Low nanomolar to picomolar Difficult and expensive to modify

1.4.3 Application of Aptamers

Aptamers as very effective tools with impressive qualities have been studied for further clinical applications in numerous investigations concerning their use as a diagnostic and therapeutic tool, bio-sensing probe, and in the development of new drugs and drug delivery systems. There have been attempts to search for aptamers that are specific to targets involved in various diseases, such as cancer and viral infection (Song, Lee, & Ban, 2012). The already developed aptamers have been studied for further applications as diagnostic or therapeutic tools.

1.4.3.1 Aptamers for target identification

In oncology, the aptamer field is rapidly expanding. Their high binding to target molecules enables detection of cancer cells, resulting in early and sensitive diagnosis. Deregulation of cell-

surface membrane protein expressions and activities are hallmarks of cancer cells. Uniquely expressed, overexpressed, or mutated surface markers in tumours are major pharmaceutical targets for therapies since personalised medicine is in rapid development (Mercier, Dontenwill, & Choulier, 2017). Advances in genomic, proteomic, and metabolomics techniques led to the discovery of tumour biomarkers including cell surface receptors (H. W. Chen, Medley, Smith, Sefah, & Shangguan, 2013; Kunii, Ogura, Mie, & Kobatake, 2011). Highly specific aptamers have been selected against a wide range of intracellular and extracellular targets such as extracellular matrix proteins (Hicke et al., 2001), cell-adhesion molecules (Dua, Kim, & Lee, 2011), chemokines (Marro et al., 2005), tyrosine kinase receptors (Ohuchi, Ohtsu, & Nakamura, 2006; Tan et al., 2013), nucleolin (D. W. Hwang et al., 2010), and many others.

It represents a great challenge to develop a biomarker system that would be able to reflect cell's physiological state with only one oncogene. Such biomarkers are specific for a certain stage of carcinogenesis and cancer development may not simply lead to an altered expression of it. The quantification of expression levels of variety of molecules within a detection platform would therefore represents beneficial information. Multiplexed systems that incorporate aptamers for a variety of proteins can be performed, and the aptamers that do bind to protein can be quantified via microarray analysis in order to determine the ratio of specific proteins (Chang, Donovan, & Tan, 2013). Such multiplexed aptamer-based proteomics has emerged as a potentially powerful analytical and quantification tool, which could complement mass-spectrometry (MS) based approaches. In such a way, several thousands of proteins can be simultaneously measured and profiled with high accuracy from a tiny amount of serum or plasma, thus becoming an attractive technique for biomarker and clinical purposes (Shangguan, Cao, Meng, Mallikaratchy, & Sefah, 2009).

1.4.3.2 Aptamer as therapeutics - clinical trials of aptamers in medicine

Aside from aptamers, other oligonucleotides have been under clinical development for the past 30 years. First were antisense oligonucleotides (ASOs) followed by and aptamers and, about 15 years ago, siRNAs. During that period of time, numerous clinical trials have been performed. Of all the molecules evaluated until 2017, six provided clear clinical benefit in rigorously controlled trials, with one aptamer among them (Stein & Castanotto, 2017). An aptamer drug, Macugen (Pegaptanib), was developed for the treatment of neo-vascular age-related macular degeneration (AMD), a disease of the eye and the leading cause of blindness in people over the age of 50. This anti-angiogenic aptamer is targeting VEGF-165, which is a homodimeric protein and the most abundant VEGF isoform that regulates vascular permeability (Ng et al., 2006).

Besides the already approved MACUGEN, there are 12 aptamers undergoing clinical trials at the moment (Table 1.2), out of them AS1411, NU172, and REG1 are the most promising. The most advanced aptamer on the potential treatment of cancer is AS1411 (known also as AGRO100), a guanine rich, nucleolin binding aptamer that is able to internalise into the cancer cells. The therapeutic benefit of AS1411 is presumably attributed to interference with intracellular pathways by disruption of nuclear factor kappa B (NFκB) signalling, thereby inducing apoptosis (Bates et al., 2017; Bates, Laber, Miller, Thomas, & Trent, 2009; Reyes-reyes, Salipur, & Shams, 2015). NU172-intravenously administered thrombin targeting aptamer prevents the formation of blood clots during acute cardiovascular surgical procedures. This aptamer is currently being evaluated in Phase II clinical trials for anticoagulation in heart disease treatments by ARCA Biopharma. ARC1779 is a DNA aptamer, truncated to 39 nucleotides and stabilized by backbone modifications, 20 kDa PEG conjugation, and 3'-capping. It blocks binding to platelet membrane glycoprotein Ib receptors, thus inducing antithrombotic effects and provides a potential therapeutic benefit in acute coronary syndromes and von Willebrand disease. Anticoagulant based on a factor IXa binding aptamer, RB006, and a complementary antidote, RB007- together known as REG1 are recently, in a phase II study for Acute coronary syndrome (ACS), and are showing promising results indicated by a lower bleeding rate (Xiaohua, Castanares, Mukherjee, & Lupold, 2012).

Table 1.2: Aptamers in Clinical Trials (Kanwar et al., 2015).

NAME	MOLECULAR TARGET	DISEASE	MODE OF ACTION	CLINICAL TRIAL PHASE
PEGAPTANIB/ MACUGEN	Vascular Endothelial Growth Factor (VEGF165)	Age-related Macular Degeneration (AMD) of the retina	Competitive inhibition of VEGF	FDA approved
REG1	Factor IXa	Arterial thrombosis	Controls blood thinning	Phase III
AS1411	Nucleolin	Acute myeloid leukemia AML	Inhibits DNA replication to cause cell death	Phase III
LY2181308	Survivin mRNA I	Non-small cell lung cancer (NSLC)	Inhibits survivin expression	Phase III
E100300/ FOVISTA	Platelet-derived Growth Factor (PDGF)	Neovascular AMD	Prevents binding of PDGF	Phase III
E ₂ F/ EDIFOLIGDE	E ₂ F	Vein graft failure of heart and leg	Inhibits the transcription factor E ₂ F and cell proliferation	Phase III
EYE001	VEGFR	Neovascular AMD	Prevents formation of new blood vessels	Phase II/III
ARC1779	A1 domain of Von Willebrand Factor (vWF)	Acute coronary syndromes	Inhibits binding of platelet receptor glycoprotein Ib	Phase II/III
Nox-E36/ EMAPTICAP PEGOL	Monocyte chemotactic protein-1 (MCP-1)	Type 2 diabetes mellitus and diabetic nephropathy	Inhibits MCP-1	Phase IIb
Nox-A12	Stromal cell derived factor-1 (SDF-1 alpha)	Various types of cancer	Inhibits SDF-1 P	Phase IIa
Nu172	Thrombin	Heart diseases	Prevents blood clotting	Phase II

ARC1905	Complement Component 5 (C ₅ Factor)	AMD	Prevents formation of membrane attack complex	Phase II
ARC19499	Anti-tissue factor inhibitor (TFPI)	Haemophilia	Improves blood clotting	Phase I/II

1.4.3.3 Aptamers and Lung Cancer

Early diagnosis is the way to improve lung cancer survival rate. Due to the lack of sensitive and selective molecular probes for lung cancer cells early diagnosis still represents a great challenge. Here aptamers could represent a new approach to achieve that goal. Research aimed at early detection, which is critical to reducing mortality and morbidity, has turned to the development of suitable aptamers. More than 20 different aptamers selected by a number of research groups all over the world demonstrated their high sensitivity for lung cancer diagnosis in different sensor systems as well as a unique potential for a targeted therapy (G. S. Zamay et al., 2015; T. N. Zamay et al., 2017).

Several aptamers against SCLC and NSCLC cell surface markers are showing promising results. The panel of selected aptamers ensures widespread availability and selectivity, despite it being selected without prior knowledge about specific biomarkers. They represent a sensitive detector for malignant cells and are able to distinguish them from normal epithelial cells while their specificity is measured in nanomolar range. Their great features remains preserved also under physiological conditions as well as after chemical fixation, suggesting that they can be applicable for *in vivo* experiments (H. W. Chen et al., 2013; Jimenez et al., 2012; Kunii et al., 2011; Zhao, Xu, Shi, Tan, & Shanguan, 2009).

Assays whether aptamers selected by Cell-SELEX can specifically recognize lung cancer cells they were done on different cell lines. The results indicate specific recognition of lung adenocarcinoma cells, one of them also recognises both squamous carcinoma cell lines. This result indicates that these aptamers can be used to distinguish different subtypes of non-small-cell lung carcinoma. In addition, these four aptamers show the differential binding to the different non-small-cell lung carcinoma patient samples, indicating that these aptamers can even distinguish molecular differences among patients with the same diagnosis (Y. Zhang, Chen, Han, Ocsay, & Tan, 2010).

Aptamers selected to postoperative adenocarcinoma tissues have been utilized for the detection of circulating tumour cells in blood, characterisation of histological structure of lung adenocarcinoma, and electrochemical sensing of blood plasma biomarkers. Aptamers to lung cancer were developed not only for diagnostics, but also for cancer treatment. Aptamers alone demonstrate antitumor activity in cell cultures. Xu and co-workers succeeded in the selection of aptamers against EGFR-transfected lung adenocarcinoma cells. They were not just showing high affinity to the cells with nanomolar dissociation constants, but also were able to internalise into cells and had good stability in serum. After entering the cells, they cause a significant cytotoxicity (Xu et al., 2013).

The great phenotypic diversity of each histological lung cancer type and the absence of highly specific and sensitive biomarkers make lung cancer diagnosis rather difficult but with the development of new aptamer approaches, we are one step closer to achieving that goal

1.4.4 Aptamers and Bioinformatics

The simulation of aptamer selection gives an insight into the selection process, including the optimal concentration ratios, the effect of background binding, and the stochastic nature of SELEX. The rising area in the field of selection simulations is aptamer binding. The development of more accurate aptamer binding models and applying them in simulations may lead to new insights into the selection process and improve its understanding.

1.4.4.1 Aptamer selection simulations

The complexity of aptamer selection is combining the range of experimental parameters and the starting combinatorial complexity of nucleic acid libraries. An Aptamer Base represents a database that provides detailed and structured information about the chosen experimental conditions for aptamer selection (Cruz-Toledo et al., 2012). The starting nucleic acid library, target choice, selection workflow, experimental conditions, and resulting aptamer affinity were statistically analysed and quantified, showing that the choice of target and selection template have the most significant impact on the selection of aptamer (McKeague et al., 2015). Combinatorial complexity of the initial library pool ($\sim 10^{15}$) makes empirical analysis of all subsequent rounds and present output sequences not achievable. Therefore, a computational simulation represents a great solution and gives an insight into possible optimisation of the SELEX protocol. The complexity of SELEX consists of the number of sequences and also individual aptamer informational properties: the sequence, folding, and target binding (Kingham, Fraser, & Tanner, 2017).

In 1991, SELEX was first simulated using a program named SELEXION (Systematic Evolution of Ligands by Exponential Enrichment with Integrated Optimization by Non-linear Analysis). The investigated properties further used for modelling of ligand binding included predicted enrichment under different conditions, optimal protein concentration depending on the dissociation constant (K_d), likelihood of selection success, and sequence representation in random pool (Irvine, Tuerk, & Gold, 1991). For investigating SELEX against multiple targets the SELEXION principle has been upgraded to program MultiSELEXION, thus capable of analysis Cell-SELEX and *in vivo* SELEX experimental data (Vant-hull, Payano-baez, Davis, & Gold, 1998). Further simulation models have focused on different SELEX parameters. When target concentration and the effect of nonspecific binding were taken into account, the higher K_d and more specific sequences resulted from higher average ratio of the starting target (Wang, Rudzinski, Gong, Soh, & Atzberger, 2012).

To overcome the discrepancy between real-life systems and computational prediction the models need to be as accurate as possible, especially the aptamer binding model.

1.4.4.2 Molecular dynamics

To improve the efficacy of aptamer selections, molecular dynamics can be predicted by computationally solving the three-dimensional structures of nucleic acids and their targets and simulating the physical forces involved in nucleic acids docking to a target. There are two possible ways to analyse the molecular dynamics - docking of whole sequence or just its fragment.

In simulating the docking between a target and an aptamer, several non-covalent interactions are assessed including ionic interactions, hydrogen bonds, van der Waal's forces, hydrophobic interactions, base stacking interactions, and shape complementarity (Setny, Bahadur, & Zacharias, 2012).

While whole sequence docking requires information about three-dimensional structures of both nucleotides and the chosen target, a fragment-based approach represents a simplified technique when only knowledge about the target structure is required. It consists of three steps: determination of the probability distribution of the first nucleotide binding to a target, then additional nucleotides are sequentially added, and finally, based on entropic criterion, the cut-off length is determined (Tseng, Mane, Kaptzy, Mercer, & Tuszynski, 2011).

1.4.4.3 Designing of libraries

Aptamers require secondary structure to bind to their targets. Increasing the occurrence of a secondary structure in the library should yield enhanced success rates of the selection and improve the binding affinity of isolated aptamers.

The presence of distinctive secondary structures, such as a stem-loop, enhances the binding affinity (Carothers, Oestreich, Davis, & Szostak, 2004; Carothers, Oestreich, & Szostak, 2006). The latter depend on the sequence nucleotide distribution. Aptamers with better binding properties tend to have more purine than pyrimidine (Knight et al., 2005). That demonstrates the importance of adjustment of base composition for further lowering the total number of candidates in a SELEX experiment and therefore a better and faster way for selecting the one with the best properties.

Computational methods use sequence information to pattern initial libraries, which results in the evolution of more complex sequences, but on the other hand reduces the size of the library. The alternative purine and pyrimidine patterning strategy has enabled the reduction of the starting library to the degree, allowing for the integration of the entire library onto the microarray.

Following the selection targeting thrombin resulted in the discovery of thrombin-specific sequences (Martin et al., 2015).

1.4.4.4 Next Generation Sequencing and *in silico* Aptamer identification

High-Throughput Sequencing (HTS) data obtained with DNA sequencing technologies are giving new opportunities for analysis of millions of sequences resulting in each selection round and consequently a new approach for identification and optimisation of aptamers (Blind & Blank, 2015; Dupont, Larsen, Jensen, Andreasen, & Kjems, 2015; Takahashi, Wu, Ho, Chomchan, & Rossi, 2016). With sequenced pools obtained after each selection round, the evolutionary dynamics of the process is monitored, therefore making identification the best sequence in early rounds very possible (Thiel et al., 2012; Valenzano et al., 2016). This also enhances our understanding of enrichment principles, selection efficiency and molecular interactions, and library convergence can be determinate. Using the HTS dynamic monitoring method, researchers successfully identified high-binding aptamers for small molecule targets. In the selection of tobramycin aptamer, HTS revealed that the best binding sequences are enriched after two selection rounds (Spiga, Maietta, & Guiducci, 2015). This data-rich approach has been also shown to be successful for selecting aptamers for proteins (Scoville, Kyu, Uhm, Shallcross, & Whelan, 2017).

1.4.4.4.1 Benchmark Toolkits for Sequence analysis

After sequencing, the large amount of DNA sequence data has to be processed and analysed. The two main steps in that procedure are pre-processing (the removal of the adapter, barcode, or constant region) and counting of sequence frequency (Hamada, 2017; Kinghorn et al., 2017). Sequence pools derived from HTS are in large number similar to each other. Therefore, scalable clustering into groups or families is an important approach for further analysis.

To date, different easy-to-use bioinformatics tools are available to analyse DNA aptamer sequences obtained by regular SELEX (Table 1.3): AptaCluster (Hoinka, Berezhnoy, Sauna, Zuben, Gilboa, & Przytycka, Teresa, 2014), AptaMotif (Hoinka, Zotenko, Friedman, Sauna, & Przytycka, 2012), MPBind (P. Jiang et al., 2014), FASTAptamer (Alam, Chang, & Burke, 2015), AptaTRACE (Dao et al., 2016), APTANI (Caroli, Taccioli, A, Serafini, & Bicciato, 2015), COMPAS (Blind & Blank, 2015), AptaMut (Hoinka, Berezhnoy, et al., 2015). Out of them, MPBind, FASTAptamer, COMPAS, AptaTrace, AptaMut, and AptaSim are capable of analysing HTS information from multiple pools.

The tools make use of different approaches, but two main characteristics used are secondary structure motif guided analysis (APTANI, AptaTrace, and AptaMotif) and sequence abundance and scalable clustering in population (AptaCluster, FASTAptamer, and APTANI). AptaSim and AptaMut are using different approaches: the simulation of HT- SELEX that includes error-prone amplification and the prediction of potential affinity achieved by altering mutants, respectively.

To make the tools even more user friendly, purely text-based tools (FastAptamer and AptaCluster) are improved with a graphical user interface (GUI) for the dynamic visualization and navigation through the data. The latter is integrated in the pipeline platform COMPAS, developed by AptaIT GmbH and the AptaGUI, an open source, platform free tool (Hoinka, Dao, & Przytycka, 2015).

MEME (Multiple Em for Motif Elucidation), a general motif-finding program (Bailey, Johnson, Grant, & Noble, 2015), is another useful tool for the identification of structure patterns from a set of aptamer candidates. Additional features for detecting the sequence motifs in a single strand loop region within the sequence is added to its extension-MEMERIS (Hiller, Pudimat, Busch, & Backofen, 2006)

Table 1.3: The list of major Benchmark Programs for SELEX data analysis. Their following characteristics are ticked off: method of sequence obtaining, further analysis approach and the final output.

	SEQUENCE GENERATION		ANALYSIS APPROACH		OUTPUT	
	SELEX	HTS	Motif Guided	Sequence Abundance	Graphical User Interface	Purely-Text Based
AptaMotif	✓		✓			✓
MPBind	✓	✓		✓		✓
AptaCluster/ AptaGUI	✓			✓	✓	
COMPAS	✓	✓		✓	✓	
FASTAptamer	✓	✓		✓	✓	
APTANI	✓		✓			✓
AptaTrace	✓	✓	✓			✓
AptaSim	✓	✓		*		✓
AptaMut	✓	✓		*		✓

*AptaSim and AptaMut are using alternative analysis approaches.

1.6 Hypotheses and Aim

1.6.1 Hypotheses

The following hypotheses were tested in this doctoral dissertation:

- Cell surface specific features of NLSC lung adenocarcinoma cell line A549 allow for selection of a set of aptamers with high selectivity for lung cancer cells, using Cell-SELEX methodology.
- Different bioinformatics tools are proposed to be used in aptamers' analysis, and we hypothesize that these may converge in the selection of the best binding aptamer sequences.
- A fraction of A549 cells express CD90, the lung cancer stem cell (LCS) marker, and we hypothesize that this subpopulation could be targeted by a newly selected set of aptamers, obtained via positive CD90⁺ cell selection step in Cell-SELEX process.

1.6.2 Aim

The major aim of this study is using Cell-SELEX methodology to develop DNA aptamers, specific for non-small lung adenocarcinoma cells, which also have cancer-stem like cell characteristics. The selection of best binding aptamer candidates is to a great extent based on application of different bioinformatics approaches, and the selected aptamer was validated by *in vitro* experiments.

Chapter 2

Materials and Methods

2.1 Cell Line

Cell culture experiments were carried out using an established human lung carcinoma cell line A549. The cell line was purchased from American Type Culture Collection (ATCC® CCL-185™, ATCC, Manassas, VA, USA).

2.1.1 Materials

Cell culture media and supplements including Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), L-glutamine and penicillin/streptomycin were obtained from Millipore Sigma (Life Technologies™, Omega, Ljubljana, Slovenia). Cell culture reagents including Trypsin-EDTA (0.05 %), Phenol Red, DMSO, EDTA, and Trypan Blue were purchased from Millipore Sigma (Life Technologies™, Omega, Ljubljana, Slovenia). Phosphate buffered saline 10× concentrate (PBS) was purchased from Sigma-Aldrich (Merck, Mikrolabo d.o.o., Maribor, Slovenia). All plasticware including tissue culture flasks, well plates with lids, centrifuge tubes, serological pipettes, unplugged Pasteur pipettes, Eppendorf tubes, syringes and membrane filters were purchased from Thermo Scientific™ (Majbert, Ljubljana, Slovenia). Primary and secondary antibodies were purchased from ABCAM (Cambridge, United Kingdom).

2.1.2 Media and Supplements

The media and supplements for the A549 cell line were in accordance with the ATCC recommendations. The media preparations were carried out under aseptic conditions. Cell culture media DMEM was supplied in 500 ml bottles, to which 50 ml of FBS, 5 ml of L-glutamine (200 mM) and 5 ml of penicillin/streptomycin were aseptically added to achieve a final concentration of 10 % FBS, 2 mM L-glutamine and 2 mM penicillin/streptomycin, respectively. The prepared media were stored at 4 °C for up to 4 weeks. Cell line was routinely grown in 75 cm² or 25 cm² tissue culture flasks and maintained in a 37 °C humidified incubator supplied with 5 % CO₂, 95 % O₂. For all the experiments in the study, each of the cell lines were harvested when they reached 70-80 % confluence and were used between passages 60-65.

2.1.3 Cell Line Authentication

The cell line authentication was performed with IdentiCell STR allele protocol and showed a 100 % match with original ATCC cell line A549 (IdentiCell, Department of Molecular Medicine, Aarhus, Denmark).

2.1.4 Routine Cell Line and Maintenance

When the monolayer of cells reached 70-80 % confluency, cells were sub-cultured to fresh flask. The growth media was aspirated and the cells were washed with 0.1 M PBS, pH 7.4 to remove any traces of serum. For maintenance cells were detached by incubation with 0.25 % trypsin diluted in 0.1 M PBS, pH 7.4 for 5 min at 37 °C in a humidified incubator supplied with 5 % CO₂,

95 % O₂. For experiments, cells were detached with 2mM EDTA pH 8, diluted in 0.1 M PBS, pH 7.4 for 15 min at 37 °C in a humidified incubator supplied with 5 % CO₂, 95 % O₂. After both, trypsin and EDTA treatment, the flask was tapped gently and viewed under the inverted light microscope to ensure the detachment of cells. Media was then added to neutralize the reaction and mixed well to obtain homogenous and evenly distributed cell suspension. The cells were seeded at the ratio of 1:4 to each fresh flask or used for experiments as stated below.

To maintain the cells of each passage for comparable experiments, cell batches were frozen down to -80 °C and if not used, transferred to liquid nitrogen. Cell cultured freezing media was prepared with supplemented growth media with L-Glutamine and DMSO. Cells at density of 5×10^5 to 1×10^6 were resuspended in 1 ml of ice cold freezing media and on ice immediately transferred to -80 °C.

2.1.4.1 Growth and Biological characteristics of Cells lines used in the study

This assessment was done using the trypan blue exclusion method. Viable cells with intact membrane excluded trypan blue and cells stained blue were classified as dead cells. Cells at a density of 6×10^4 cells/ml were then seeded into T-25 flasks and incubated at 37 °C supplied with 5 % CO₂, 95 % O₂. At time points between 2 and 5 days, cells were detached and resuspended in media prior to a 1:1 dilution of cell suspension and Trypan Blue. The number of viable cells were counted using a hemocytometers and light microscope (x10 magnification). The average viable cell count per square (n=5 squares) was multiplied by the hemocytometer factor (10^4) and the dilution factor (x2) to give number of viable cells/ml.

$$\begin{aligned} \% \text{ viable cells} &= [1, 00 - (\text{Number of blue cells} \div \text{Number of total cells})] \times 100 \\ \text{Cells/ml culture} &= \text{Number of viable cells} \times 10^4 \times 2 \end{aligned} \quad (2.1)$$

When cell viability should was at least 95 % for healthy log-phase, the cells were used for further maintenance or experiments. The surface area, seeding density and plating volume for each culture flask or plate is given in Table 2.1. Non-plated cells, to be used for experiments were kept on ice.

Table 2.1: Cell culture plating.

	SURFACE AREA (cm ²)	SEEDING DENSITY (number of viable cells)	VOLUME OF GROWTH MEDIUM (ml)
Culture Flask			
T-75	75	2.1×10^6	8-15
T-25	25	0.7×10^6	3-5
Culture Plate			
6-well	9	0.3×10^6	3-5
12-well	4	0.1×10^6	1-2
24-well	2	0.05×10^6	0.5-1.5

2.2 Oligonucleotide Synthesis

A nucleotide library containing 34 random bases (N34) was chemically synthesized and purified using PAGE by Eurofins (Hamburg, Germany). Letter N in the library indicates the random region of where any of the 34 deoxy nucleotides occurs with approximately equal probability.

The N34 sequence was conjugated with FAM (Fluorescein Isothiocyanate) at 5'- binding site as follows: 5'-FAM-GCCTGTTGTGAGCCTCCT-N34-CGCTTATTCTTGTCTCCC-3'.

The oligonucleotides, used for further binding experiments (listed in Table 2.2) were synthesized by Microsynth AG (Balgach, Switzerland).

Table 2.2: List of oligonucleotides used with their chemical features.

SEQUENCE NAME	PURIFICATION	MODIFICATIONS	LENGTH (number of nucleotides)	MELTING POINT*	MILIMOLAR EXTINCTION COEFFICIENT	MOLECULAR WEIGHT (g/mol)
A316_5	PAGE	5'FAM	70	68.8	647.8	21 900.2
	5'-GCC TGT TGT GAG CCT CCT GCA TCC ATT AGT TAT TGT TTA TTT TGA TAT TAT ACG CTT ATT CTT GTC TCC C-3'					
A452_3	PAGE	5'FAM	69	69.9	637.2	21 463.6
	5'-GCC TGT TGT GAG CCT CCT ACA CAC TTT GAA CTT ATA ACT CTA TTC TCT ATA CGC TTA TTC TTG TCT CCC-3'					
A288_7	PAGE	5'FAM	70	79,0	625,5	21 801.7
	5'-GCC TGT TGT GAG CCT CCT CCA CCC CGA CTC AAG GGA ACC GGT CGG CCT GCC CCG CTT ATT CTT GTC TCC C-3'					
A218_12	PAGE	5'FAM	70	69.6	633.2	21 852.2
	5'-GCC TGT TGT GAG CCT CCT GTG TGT ACT TAT CCT ATT TTG TTT ACC TAT ATT ACG CTT ATT CTT GTC TCC C-3'					
A254_11	PAGE	5'FAM	70	68.8	649.1	21 900.2
	5'-GCC TGT TGT GAG CCT CCT GCA CTA TCT AGT TAT TGT TTA TTT TGA TAT TAT ACG CTT ATT CTT GTC TCC C-3'					
A452_3	PAGE	5'FAM	69	69,9	637.2	21 463.6
	5'-GCC TGT TGT GAG CCT CCT ACA CAC TTT GAA CTT ATA ACT CTA TTC TCT ATA CGC TTA TTC TTG TCT CCC-3'					
A155_18	PAGE	5'FAM	70	93	616.9	21 801.7
	5'-GCC TGT TGT GAG CCT CCT GCC AGT TCC GGA ACC CTG CGT CGG GTG CGT CCT CCG CTT ATT CTT GTC TCC C-3'					
S6	PAGE	5'FAM	85	93.5	657.1	27 022.6
	5'-ACG CTC GGA TGC CAC TAC AGG TGG CCA GTC ACT CAA TTG GGT GTA GGG GTG GGG ATT GTG GGT TGC TCA TGG ACG TGC TGG TGA C-3'					

2.3 *In vitro* Selection

Aptamer selection was performed on target A549 cell line suspension. Cells were detached by incubating with 0.02 % EDTA in ice cold 1x PBS, at 37 °C µl for 15 min and filtered through 70 µm sieve. Cells were then washed twice with 500 µl of ice cold 1x PBS and resuspended in 500 µl of ice cold 1x binding buffer (Table 2.3).

Table 2.3: Composition of 1x binding buffer (pH=7.4).

	Concentration
HEPES	25 mM
KCl	5.4 mM
CaCl ₂	2.8 mM
MgCl ₂	3.2 mM
NaCl	144 mM

The initial pool containing 5 nmol of DNA library was dissolved in 500 μ L of binding buffer. The library was folded by heating at 95 °C for 20 min, followed by snap cool on ice for 10 min. The DNA library was then kept at 25 °C for 20 min prior to the incubation with target cells. Incubation with 300 000 target cells was performed in a microcentrifuge tube for 30 minutes at 25 °C with gentle shaking (600 g). After incubation, the cells were washed twice with 500 μ L of ice cold binding buffer. After every washing step, cells were pelleted by centrifugation 600 g for 5 min and supernatant was gently removed.

The incubation of cells with the aptamers was followed by the BRASIL technique for separation of unbound from oligonucleotides bound on cells (Giordano, Cardó-Vila, Lahdenranta, Pasqualini, & Arap, 2001). With centrifugation of a mixture of cells and oligonucleotides through the dibutyl phthalate: cyclohexane (9:1 [v: v]; d=1.03 g/ml). The cells with bound aptamers were collected from the pellet of the organic phase and separated by centrifugation (13 000 g for 10 min (Hettich Universal 32R centrifuge, HETTICH Instruments LP, Beverly, MA, United States). The pellet containing aptamers was extracted by phenol/chloroform to obtain nucleotides for next selection round.

For enrichment of bound pool, a PCR reaction followed by separation of ssDNA on polyacrylamide denaturing gel was carried out. The bands showing FAM fluorescence on transilluminator were carefully cut out from the gel and extracted in microcentrifuge tubes. To restore a sufficient amount of DNA and final 0.4 μ M ssDNA for the next selection round it was necessary to perform PCR reaction.

2.3.1 Negative Selection with Human Blood Cells

The negative selection was introduced in the 7th selection round using 3.5 μ L human blood cells in 1x PBS (erythrocytes, leukocytes, and thrombocytes). Cells were dissolved in 500 μ L of binding buffer were incubated with 1 μ M of ssDNA pool from previous selection round and followed by a positive selection with A549 cells. Blood cells were separated from plasma by density gradient separation with Ficoll-Paque (Miltenyi Biotec, Bergisch Gladbach, Germany). 5 ml fresh anticoagulated blood was diluted with 3x volume of 1x PBS buffer. In 50 ml conical tube diluted cell suspension was carefully layered with 15 ml of Ficoll-Paque. Cell suspension was centrifuged at 400 g for 35 min at 20 °C in a swinging bucket rotor (SX4400 Swinging-Bucket Rotor, Beckman Coulter, Brea, United States). After centrifugation the upper layer with blood plasma was carefully removed. Remaining cells were washed once with 15 ml of 1x PBS buffer and centrifuged at 600 g for 5 min. Supernatant was removed and cells were resuspended in 5 ml of fresh 1x PBS buffer.

2.3.2 Sorting of Cell Population Positive for CD90

For cell preparation for cell sorting the same procedure as for previous positive selection rounds was followed (described in 2.3). Additional step of labelling A549 cell population with CD90 antibody (AntiCD90/Thy1 PE/Cy5; ab95698; Abcam; Cambridge, United Kingdom) was introduced after incubation of cells with aptamers. Gate was determined to collect the double-labelled cells with both- CD90 antibody and aptamer oligonucleotides. Cell sorting was used to isolate the identified fraction of cells. A fraction of 2 500 cells was successfully collected and aptamers were extracted from cells and purified for PCR amplification.

2.4 Sequencing and Bioinformatics Analysis of Aptamer Pools

2.4.1 Sanger Sequencing

Selected aptamer pool obtained in each selection round was tested using Restriction Fragment Length Polymorphism analysis (RFLP) for the presence of conserved restriction sites. Different bands were obtained that represented fragments of DNA with conserved restriction sites, thus showing selective enrichment of specific groups of oligonucleotides during each round of SELEX. Out of seven rounds, the obtained ssDNA from final three was used for sequencing using the DNA pJET 1, 2 plasmid (Fermentas Kit #K1213) and *E. coli* cells competent for OneShot TOP10. 64 colonies from each pool were collected and sequenced.

2.4.2 Next Generation Sequencing

The 7th SELEX aptamer pool was sequenced by Ion Torren PGM technology. The Torrent suite software provided FASTQ output file, which comprised raw sequences without adaptor and barcode sequences.

2.4.3 Bioinformatics Analysis

2.4.3.1 Bioinformatics analysis using CLC genomics workbench software

In the first selection procedure obtained by FASTQ, the file was exported to CLC Genomics Workbench Software 9.5.3. (<https://www.qiagenbioinformatics.com/>) for further processing. Raw data was quality filtered by length and DNA orientation. Library primers were trimmed. Reads that were absent in copies were abandoned. The remaining set of sequences was decimated to 10 % and unique sequences were identified. These were blasted against the initial pool of quality filtered reads to obtain clusters of sequences with at least 96 % sequence homology. Representative sequences from 16 most abundant clusters were analyzed for presence of conserved sequence motifs by MEME tool (Bailey et al., 2009)

2.4.3.2 In house benchmark method of bioinformatics selection

In the second selection procedure reads we used Shell Script to firstly quality filtered by base composition followed by selecting reads having only exact library primer sequence, all others were abandoned. Remaining reads were sorted according to copy number. Twenty most abundant sequences were manually inspected for properties of their computed foldings using UnaFold tool (Markham and Zuker, 2008).

2.4.3.3 COMPAS Benchmark Toolkit sequence analysis

The third aptamer selection procedure was based on COMPAS software (AptaIT GbmH; Planegg, Germany). This bioinformatics approach identify ligands based on co-occurrence mode. It identifies ligands in context of sequence families (patterns). Non-identical sequences that contain two frequent motifs are grouped into sequence families. The defined degree of similarity was set on 80 %. After absolute and relative number of presence for each candidate was calculated, 12 most frequent families was chosen for further analysis based on melting temperature (T_m) and number of sequence repetitions in total aptamer pool.

2.5 Determining the Binding Affinity of the Aptamers to the Target A549 Cells by Flow Cytometry

Oligonucleotide pools (library, 6th and 7th round of selection) were tested on A549 cells. Pools were incubated in binding buffer for 20 min at 95 °C, then immediately placed on ice for 10 min and maintained at 25 °C for 20 min. For the interaction between single-stranded DNA molecules and cells, the solution containing the aptamers was gently mixed with cells (10^6 cells/microtube) to a final volume of 100 μ l and a 30 pM concentration of aptamers. Untreated cells used as control were incubated with binding buffer alone. The mixture was incubated for 30 min at 25 °C with gentle shaking (Eppendorf Thermomixer, Sigma Aldrich, Darmstadt, Germany). After shaking

cells were centrifuged at 600 g for 5 min, and the supernatant discarded. A wash was performed with binding buffer, the mixture was again centrifuged and the supernatant discarded. Then 500 μ l of binding buffer was added and followed by a flow cytometry analysis.

Fluorescence analysis was performed on MACSQuant® Analyzer 10 (Miltenyi Biotec, Bergisch Gladbach, Germany). The FITC positive cells were detected with excitation using blue laser at 488 nm and filter at 525/50 nm. 30 000 events inside cell-determinate gate were recorded for each sample. The count of labelled cells was analyzed using FlowJo software (FlowJo LLC, Ashland, Oregon).

2.5.1 Effect of Different Cell Detachment Approaches on Aptamer Binding

To determine whether the different approaches of cell preparation can lead to changes of cell surface and therefore to the entry of the aptamers and unwanted positive fluorescence signal, we have tested three different approaches of cell detachment.

A549 cells used for the study were separated into three groups, each growing in separate T-25 culture flask. Detachment of cells with 0.02 % EDTA was following the cell preparation protocol described in 2.3. For trypsin detachment, after washing 0.25 % trypsin-EDTA was added to cells, those were further incubated at 37 °C and 5 % CO₂ for 5 min. The trypsin activity was terminated with addition of cell growth media. For mechanical detachment, after washing fresh cell growth media was added to cells and we gently collected them using scraper. Detached cells were collected in respective microcentrifuge tubes, incubated with aptamers and further analyzed as described in 2.4.

2.5.2 Effect of Increased Concentration of Protease Trypsin on Aptamer Binding

The experiment to provide idea about the nature of aptamer target was performed with five different concentrations of protease trypsin. The A549 cells were prepared in five separated T-25 culture flasks. After washing, cells were treated with different trypsin concentration with 0.02 % EDTA. As control only 0.02 % EDTA was used (as used for selection, described in 2.3.). Tested concentrations of trypsin added to 0.02 % EDTA were: 0.01 %, 0.05 %, 0.1 % and 0.25 %. For detachment cells were incubated at 37 °C and 5 % CO₂ for 15 min. The trypsin activity was terminated with addition of cell growth media. For mechanical detachment, after washing fresh cell growth media was added to cells and we gently collected them using scraper. Detached cells were collected in respective microcentrifuge tubes, incubated with aptamers and further analyzed as described in 2.4.

2.5.3 Effect of Incubation Temperature on Aptamer Binding

To obtain an idea if our best binding aptamer candidate binding properties changes when incubated with cells at different temperatures and therefore effect their further applications we tested binding at different temperatures. Cells were separated into three groups for the experiments and incubated with each aptamer (100 nM) at 4 °C, 25 °C and 37 °C simultaneously for 30 min. After incubation, the cells were prepared following the same protocol for flow cytometry analysis in the section 2.4.

2.5.4 Effect of Cell Passage Number on Aptamer Binding

This experiment was performed to see, if binding site of the aptamers on cell is lost with increasing passage numbers or the binding epitope remains significant.

Cells passage numbers 60, 65 and 70 were prepared in three separated T-25 culture flasks. After washing cells were simultaneously incubated with aptamers and binding recorded as described in 2.3 and 2.4.

2.6 Determining the CD90 Protein Expression on the Target A549 Cells by Flow Cytometry

As selected candidates were obtained from the pool of oligonucleotides that was bound to A549 cells expressing CD90 protein, we wanted to check its expression in A549 cells after following the same cell preparation as used for aptamer binding experiments. Flow cytometry was performed as described above. 5 μ l of antibody AntiCD90/Thy1 (PE/Cy5) (ab95698, Abcam, Cambridge, UK) was added to 0.3 x10⁶ cells resuspended in 95 μ l of ice cold 1x binding buffer.

Mouse IgG1, kappa monoclonal (PE/Cy5) (ab67435, Abcam, Cambridge, UK) Isotype Control was used for flow cytometry experiments. 10 μ l of IgG1 was added to 0.3×10^6 cells resuspended in 90 μ l of ice cold 1x binding buffer. Cells were incubated at 4 °C for 30 min with gentle shaking (Eppendorf Shaker). After shaking cells were centrifuged at 600 g for 5 min, and the supernatant discarded. Cells were washed three times with 500 μ l and resuspended in 500 μ l ice cold 1x binding buffer for following flow cytometry analysis.

Fluorescence analysis was performed on MACSQuant® Analyzer 10 (Miltenyi Biotec, Bergisch Gladbach, Germany). The PE/Cy5 positive cells were detected with excitation using yellow laser at 561 nm and filter at 661/20 nm. 30 000 events inside cell-determinate gate were recorded for each sample. The count of labelled cells was analyzed using FlowJo software (FlowJo LLC, Ashland, Oregon).

2.6.1 Double Labeling of Cells with Anti CD90 Antibody and Selected Aptamers

As result of previous binding experiments with Antibody CD90 and Aptamer candidate A155_18 gave us quite similar percentage of binding to target cell line, we decided to test ligand competition binding, to test if both of the ligands are binding to the same target.

As the base for binding protocol we were following the Ligand-Guided Cell-Selection protocol, reported by Zumrut et al. (Nucleic Acid Therapeutics, 2016). We decided to test three different competitive approaches: incubation of cells first with antibody, following with aptamer incubation; first incubation with aptamer, following the incubation with antibody and incubation with both at the same time. For experiment, cells were detached with 0.02 % EDTA and filtered through 70 μ m sieve. The schematic workflow of the experiment is shown in Table 2.4. As control we incubated cells only with antibody and with aptamer A155_18 respectively. To detect both of the ligands in the same flow cytometric measurement, they were carrying fluorophores detectable with different bandpass filters.

Table 2.4: Workflow of double labeling A549 cells with Aptamer A155_18 and Antibody CD90.

APTAMER → ANTIBODY	ANTIBODY →APTAMER	ANTIBODY + APTAMER
300 000 cells + 0.5 μ M Aptamer	300 000 cells + 0.025 mg/ml CD90 antibody (dilution 1:200)	300 000 cells + 0.025 mg/ml CD90 antibody + 0.5 μ M Aptamer
Washing step	Washing step	
Cells + 0.025 mg/ml CD90 antibody	Cells + 0.5 μ M Aptamer	

2.7 *In vitro* and *in silico* Comparison of Published and our Selected Aptamer

To compare binding properties of our selected best binding aptamer candidate A155_18 we performed *in vitro* and *in silico* experiments with already published aptamer S6 (Shi et al., 2013), selected against the same cell target- A549 cell line.

2.7.1 *In vitro* Comparison of Binding

Published aptamer S6 was based on its sequence synthesized by Microsynth AG (Balgach, Switzerland):

5'-FAM-GTG GCC AGT CAC TCA ATT GGG TGT AGG GGT GGG GAT TGT GGG TTG-3'.

Its original Cy5 fluorescent dye was changed with 5'-FAM as attached to our aptamers. For flow cytometry binding experiments we were following the same protocol as described in 1.4.

2.7.2 *In Silico* Comparison of Aptamer Sequence Similarities

For the sequence folding simulation and ΔG calculation we have used Unafold computer program (<http://eu.idtdna.com/unafold/Home/Index>) using energy rules: DNA, 25 °C, 14.4 mM NaCl, 3.2 mM MgCl.

For dendrogram plotting MEGA program was used: sequences were aligned with ClustalW and dendrogram was plotted with the method of maximum likelihood and the substitution model Tamura-Nei (bootstrap 1 000).

Chapter 3

Results

3.1 *In vitro* Selection of Aptamers using Cell-SELEX Method

We aimed to develop aptamer probes targeting lung cancer circulating tumor cells due to their potential diagnostic, prognostic and predictive capacity. To develop cell specific aptamers against them, we have chosen the most commonly used human NSCLC lung adenocarcinoma A549 cells as the target for Cell-SELEX. Human blood cells were adopted as a negative control for Cell-SELEX to increase the selectivity of generated aptamers exclusive to the A549 cells.

In the selection process, the cultured A549 cells were first incubated with a 70-base synthetic single stranded DNA library. The DNA sequences that bound to the A549 cells were then eluted and separated with a BRASIL technique, as described in Material and Methods.

3.1.1 Analysis of Cell-SELEX Pool Enrichment

We monitored the gradual enrichment of aptamers during the selection process by flow cytometry. The increase in the fluorescence intensity of the dye labelled DNA pools bound to target cells was gradual and steady during the progress of selection, indicating a successful evolution of high affinity aptamers. After seven rounds of selection, the binding ability of selected DNA pool reached a plateau, and cloning was performed to isolate individual aptamers. To monitor the evolution of the aptamer pool, the appearance of restriction sites in the population was analyzed by restriction fragment length polymorphism (RFLP). This revealed the emergence of distinct families in the library. The PCR product of each round of selection was digested with a mix of four restriction enzymes Alu I, Rsa I, Hae III and Hinf I. Following ethanol precipitation, the digested samples were loaded onto denaturing polyacrylamide gel (Figure 3.1), showing a reduced pattern of bands following 6 rounds of selection, suggesting that the random pool has evolved into a homogenous fraction of aptamers.

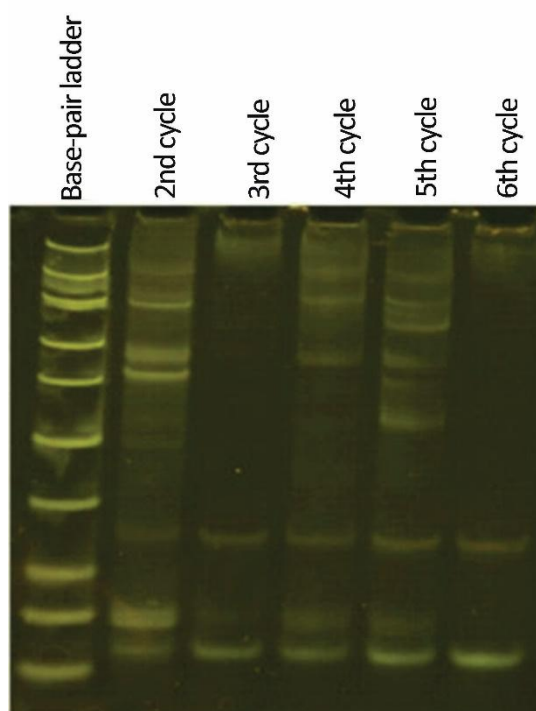


Figure 3.1: Separation of aptamer pool after 6th round of selection on a non-denaturing polyacrylamide gel. An Ultra-Low Range base-pair ladder (Thermo Scientific) was used for visualization of bands following ethidium-bromide staining.

We have compared aptamer pools from the original library, the 6th round and the negative (7th) round of selection. Regarding the percentage of labelled cells for each pool, no significant difference was observed. We observed that the population of cells that were positive for the aptamer pool from negative selection had higher fluorescence, as shown by the shift to the right in the density plot and the overlap of the histograms (Figure 3.2.A). Furthermore, we performed flow cytometry for CD90 expression in A549 cells and we observed a well-defined population that was positive for CD90 and aptamers (Figure 3.2.B). This population of cells was sorted out and bound aptamers were eluted and amplified by PCR. The obtained aptamer pool was used for further sequencing in order to find the best binding candidate.

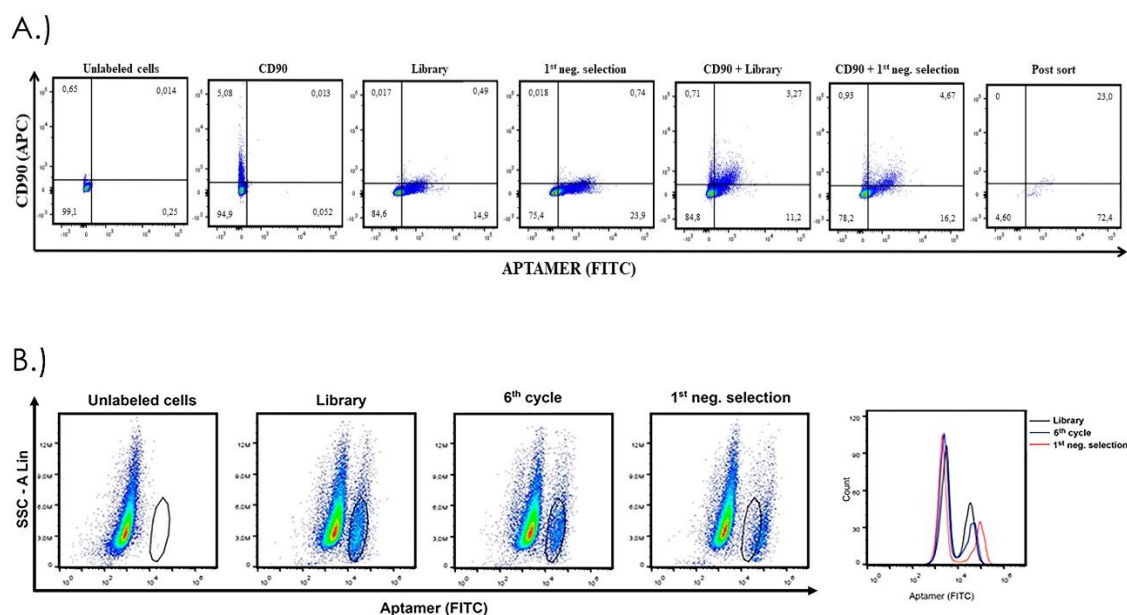


Figure 3.2: (A) Flow cytometry was performed for FITC-labelled aptamer pools comparing binding to A549 cell binding of the pool of the 1st cycle), the 6th cycle and negative selection cycle against blood cells. The density plots are showing that there is no difference in the percentage of labelled cells for each of the pools. Displacement of the population in the density plot and shift of position on the histograms for the cells labelled with the pool following the negative selection against blood cells is observed, showing a cell population with increased fluorescence intensity compared to cells labelled with the initial library or pool of 6th cycle. (B) CD90 binding and cell sorting: Adjustment of cellular gates for un-labelled cells, CD90+, Library and negative selection. Double-labelled cells CD90/library and CD90/negative selection were compared, and a gate was set to collect the cells most intensely labelled with the aptamer pool following negative selection. To confirm the profile of the isolated population, the sorted cells were analyzed again by flow cytometry (post sort). The density plots show the presence of a well-defined secondary population that is double labelled, suggesting that aptamers are recognizing a sub-population of CD90 positive stem cells in the A549 cell population.

3.2 Sequencing and Bioinformatics Analysis of Enriched Pool

The sequencing of 7th selection round pool by Ion Torrent technology resulted in 239 713 reads, 151 814 reads being unique. *In silico* selection of aptamers from the NGS data was accomplished using three different procedures as described below.

3.2.1 Bioinformatics Analysis using CLC Genomics Workbench Software

The starting pool of 239 713 reads was narrowed down to 16 representative sequences which were subjected to further selection based on the presence of conserved sequence motifs. The selection process started by filtering sense DNA sequence reads that were 34 ± 1 bases long followed by trimming library primers resulting in 135 576 high quality reads. 72.1 % reads were repeated only once and were therefore abandoned from the analysis. Number of reads with at least one copy (37 821 reads) were further narrowed down to 10 % (3 782 reads). The unique sequences (2 631 reads) were aligned against the starting pool of sequences (135 576 reads) and were clustered based on 95 % sequence homology. Eighteen representative sequences from the clusters having the highest number of sequence members, ranging from 1 080 to 142 copies per representative sequence, were subjected to selection based on presence of conserved sequence motifs. Each of

aptamers A791_1, A1080_2 and A420_8 were predicted as best binding candidates, since each of them carries a unique sequence motif (Table 3.1).

Table 3.1: List of selected aptamer sequences based on the CLC Genomics Workbench software. Sequences presented in bold were predicted as best binding ones considering number of copies in trimmed high quality reads and conserved sequence motifs.

Name of aptamer sequence	No copies in 135 576 sample		sequences identified as duplicates 37 821	all sequences 135 576
	Number of hits	Lowest E-value	%	%
A1080_2	1 080	2.14E⁻⁶	2.9	0.8
A880_4	880	9.21E ⁻⁷	2.3	0.6
A791_1	791	4.56E⁻⁶	2.1	0.6
A787_3	787	2.14E ⁻⁶	2.1	0.6
A625_5	625	2.14E ⁻⁶	1.7	0.5
A611_6	611	2.14E ⁻⁶	1.6	0.5
A554_11	554	2.14E ⁻⁶	1.5	0.4
A550_7	550	2.14E ⁻⁶	1.5	0.4
A465_10	465	2.14E ⁻⁶	1.2	0.3
A421_9	421	2.14E ⁻⁶	1.1	0.3
A420_8	420	2.14E⁻⁶	1.1	0.3
A362_15	362	2.14E ⁻⁶	1.0	0.3
A338_12	338	2.14E ⁻⁶	0.9	0.2
A311_13	311	2.14E ⁻⁶	0.8	0.2
A306_14	306	2.14E ⁻⁶	0.8	0.2
A166_17	166	2.14E ⁻⁶	0.7	0.2
A155_18	155	2.14E ⁻⁶	0.5	0.2
A142_16	142	2.14E ⁻⁶	0.4	0.1

When the same motif or motif pattern was present, the cluster member with highest number of repetitions was selected. Graphical presentation of motif occurrence obtained with MEME 4.12.0 (Multiple Em for Motif Elucidation (Bailey et al., 2015)) in 18 most abundant sequences inside sequenced pool of aptamers is presented in Figure 3.3.

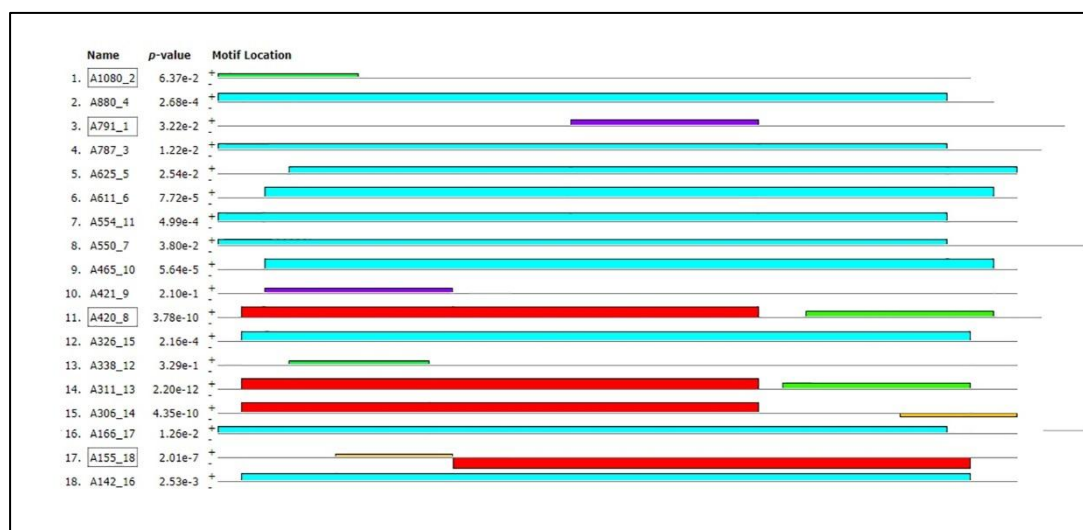


Figure 3.3: MEME search results for motif locations in selected sequences. Each motif is presented with different color rectangle, where straight line presents sequence. The position of motif is either sense- above the line, or antisense-below the line. (Selection criteria: normal mode, any number of repetition of motif per sequence, minimum motif sites:2, minimum motif width:5, maximum motif sites: 50, maximum motif width:50) (Bailey et al., 2015).

3.2.2 In House Benchmark Method of Bioinformatics Selection

In the second *in silico* aptamer selection procedure reads having ambiguous bases, incorrect or no sequence of the library primers were firstly filtered out. In the remaining high quality sequence set 80 312 reads were unique and 81.7 % reads appeared in the set only once (no copies). Twenty sequences with the highest numbers of copies (reads), ranging from 786 to 138 copies, were ranked with regard stability of the computed folding, using ΔG (kcal/mol) as a measure of stability, and stability of putative loop in first three most stable computed foldings as given by UnaFold tool (Unified Nucleic Acid Folding and hybridization package, (Markham & Zuker, 2008)).

For each of chosen parameters we scored the sequence with either 1(if true) or 0 (if not true). In addition to overall scoring summary, we have checked for presence of motifs. The final selection of aptamers was therefore the best scored candidate inside each motif group.

Aptamers A155_18, A452_3, A373_4, A218_12 were selected as candidates for further *in vitro* validation (Table 3.2).

Table 3.2: List of selected aptamer sequences based on the second bioinformatics approach. According to the scoring values for each of four criteria and presence of different motifs, four candidates (A542_3, A373_4, A218_12 and A155_18) were predicted to have best binding features.

Name of aptamer sequence	Number of repetitions above 250	ΔG (kcal/mol) under -1	ΔG (kcal/mol) under -1 without primers	ΔG (kcal/mol) with primers	Total scores	Presence of motif
A786_1	1	0	0	0	1	/
A574_2	1	0	1	0	2	motif 2
A452_3	1	1	1	1	4	motif 3
A373_4	1	0	1	1	3	motif 2
A316_5	1	0	0	0	1	motif 2
A290_6	1	0	1	0	2	/
A288_7	1	1	0	0	2	motif 3
A278_8	1	0	0	0	1	motif 2
A266_9	1	0	0	0	1	motif 2
A254_10	1	0	1	0	2	/
A245_11	0	1	0	0	1	motif 1
A218_12	0	1	1	1	3	motif 2
A217_13	0	0	0	0	0	/
A190_14	0	0	1	0	1	motif 1
A185_15	0	1	0	0	1	motif 1
A176_16	0	0	0	0	0	motif 2
A166_17	0	0	0	0	0	motif 2
A155_18	0	1	1	0	2	motif 1
A147_19	0	0	0	0	0	motif 2
A138_20	0	0	0	0	0	motif 2

3.2.2.1 COMPAS Benchmark Toolkit Sequence Analysis

Third *in silico* selection procedure was executed using software COMPASS that has integrated multiple bioinformatics tools as described previously by Blank (Blind & Blank, 2015) (AptaIT GbmH, Planegg, Germany). Here the total reads of last selection pool were firstly clustered in 998 families with defining pattern in loop space region (Figure 3.4). Each family is further represented with sequence (one with highest number of repetitions in the pool of total reads).

Selection of 21 most frequent families was followed by deconvolution grouping families into clans. The latter was depending on loop space similarity. If loop is frequently present, that indicates its relevance and thus its importance. For data analysis CompasViewer program was used.

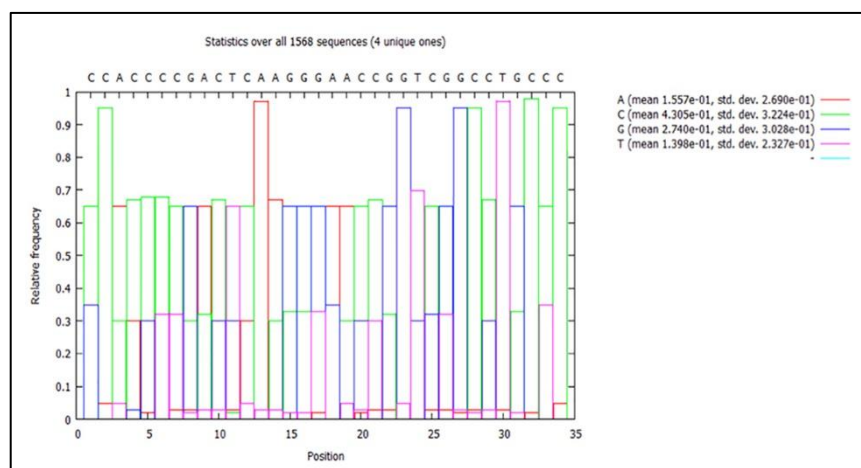


Figure 3.5: Relative frequency of nucleotides in loop space over 1 568 sequences containing Pattern 21.

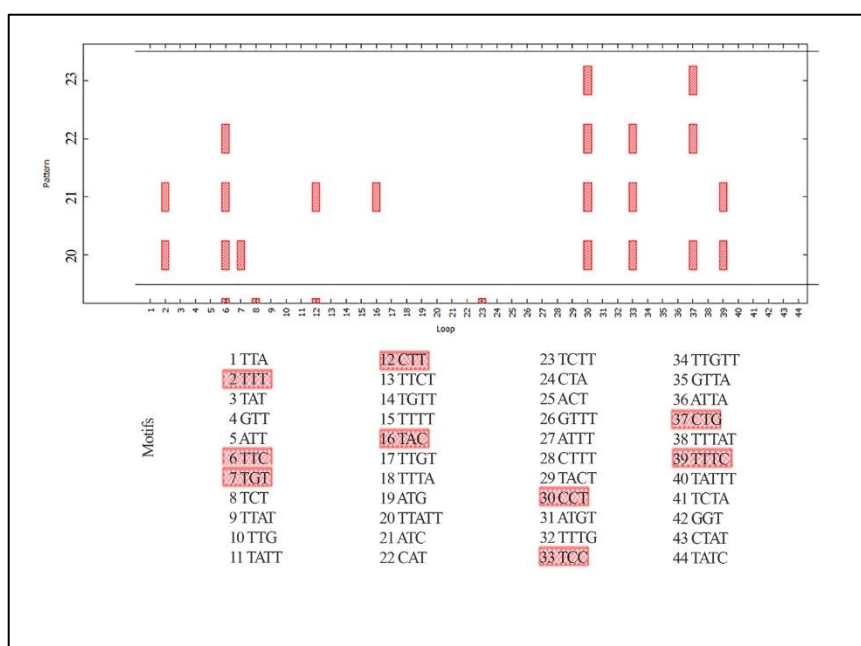


Figure 3.6: Sharing motifs occurring inside loop space of the patterns 20, 21, 22 and 23. Each motif is shown by its occurrence in potential loops in patterns sorted by absolute number of occurrences in loops. Based on the motif composition of patterns and therefore their similarity, they are composing the same clan.

Further scoring for structure stability was applied in terms of melting temperature in addition to pattern ranking (Table 3.3). For melting temperature we calculated the average and the highest possible for each Pattern. Values ranging from 300.2–354.6 for average T_m and 300.2 – 348.3 for highest T_m were divided in six groups with equal numbers of patterns with different T_m . Group with highest average T_m or overall highest T_m was given the highest score (6), with decreasing score value (1 – 5) for further respective groups. The same scoring principle was applied for Pattern ranking, where pattern ranking number represents number of repetitions of same pattern. More unique patterns (with lower repetition number; 3–21) were given the highest (6) score and patterns with the highest number of repetitions (602 – 805) were given the lowest (1) score.

Table 3.3: Scoring of the Patterns according to their average or highest melting temperature and their ranking in pool of all obtained sequences.

SCORE	Melting Temperature (Tm)		Pattern Ranking
	Average Tm	Highest Tm	
1	300.2 – 302.5	300.2 – 302.9	602 – 805
2	302.6 – 303.8	303.1 – 305.6	322 – 595
3	304.0 – 307.1	305.7 – 309.6	102 – 319
4	307.5 – 310.7	309.7 – 314.7	57 – 59
5	311.5 – 319.6	314.8 – 324.0	32 – 43
6	319.8 – 345.3	324.1 – 348.3	3 – 21

Based on the scoring, the best Patterns were selected for each of ten clans (Table 3.4). With regards of sharing the same total score value for clans 4 and 5 two patterns were selected as best ones. From all analyzed patterns, Pattern_21 has given us the best overall scoring. After the analyzing sequences presenting each pattern, seven sequences were identified as sequences recognized with previous bioinformatics approach, three of them even with potential best binding properties (Table 3.5). The schematic workflow of used COMPAS approach is presented in Figure 3.7.

Table 3.4: Scoring of the Patterns inside each of 10 clans. The scores given were according to Tm ranking and Pattern ranking. Based on total score summary, the best Pattern in each clan was selected (shown in bold).

STRUCTURE		Tm Ranking	Pattern Ranking	Total score	STRUCTURE		Tm Ranking	Pattern Ranking	Total score
CLAN1	Pattern_3	1	6	7	CLAN8	Pattern_654	1	0	1
	Pattern_43	2	4	6		Pattern_697	1	0	1
	Pattern_403	1	0	1		Pattern_95	1	2	3
	Pattern_464	2	0	2		Pattern_16	2	4	6
	Pattern_415	6	0	6	CLAN9	Pattern_652	4	0	4
CLAN2	Pattern_4	1	6	7		Pattern_718	1	0	1
	Pattern_319	1	0	1		Pattern_602	6	0	6
	Pattern_138	4	1	5		Pattern_908	2	0	2
	Pattern_553	1	0	1		Pattern_812	1	0	1
CLAN3	Pattern_439	2	0	2		Pattern_19	1	4	5
	Pattern_619	4	0	4		Pattern_772	1	0	1
	Pattern_21	6	6	12		Pattern_136	2	1	3
	Pattern_6	4	4	8		Pattern_595	1	0	1
CLAN4	Pattern_8	4	6	10		Pattern_913	4	0	4
	Pattern_14	6	4	10		Pattern_130	4	1	5
	Pattern_57	6	2	8		Pattern_574	2	0	2
CLAN5	Pattern_186	1	1	2		Pattern_844	4	0	4
	Pattern_416	2	0	2		Pattern_674	2	0	2
	Pattern_557	1	0	1		Pattern_834	1	0	1
	Pattern_123	1	1	2		Pattern_363	1	0	1
	Pattern_332	1	0	1		Pattern_494	1	0	1
	Pattern_187	2	1	3		Pattern_622	4	0	4
	Pattern_452	6	0	6		Pattern_506	1	0	1
	Pattern_378	2	0	2	CLAN10	Pattern_322	1	0	1
	Pattern_10	4	6	10		Pattern_471	6	0	6
	Pattern_466	2	0	2		Pattern_254	1	1	2
	Pattern_273	4	1	5		Pattern_681	4	0	4
	Pattern_613	4	0	4		Pattern_920	1	0	1
	Pattern_943	1	0	1		Pattern_929	1	0	1
	Pattern_37	6	4	10		Pattern_32	1	4	5
CLAN6	Pattern_791	1	0	1		Pattern_918	2	0	2
	Pattern_872	1	0	1		Pattern_379	4	0	4
	Pattern_277	4	1	5		Pattern_20	1	4	5
	Pattern_13	6	4	10		Pattern_593	4	0	4
CLAN7	Pattern_278	1	1	2		Pattern_838	4	0	4
	Pattern_805	1	0	1					
	Pattern_317	4	0	4					
	Pattern_423	2	0	2					
	Pattern_15	1	4	5					
	Pattern_102	2	1	3					
	Pattern_975	1	0	1					

Table 3.5: Selected aptamers with *in silico* Approach 2 and COMPAS approach, sharing the same sequences. All seven were recognized with both approaches, while three marked with red color were also best scored twice.

Approach 2	COMPAS
A452_3	Pattern_3
A316_5	Pattern_4
A155_18	Pattern_21
A185_15	Pattern_14
A288_7	Pattern_10
A218_12	Pattern_15
A254_11	Pattern_16

3.3 Characterization of Aptamers

After bioinformatics analysis, a representative four sequences were chosen to test their binding abilities against target cell line A549: A452_3, A155_18, A218_12 and A373_14 using flow cytometry.

3.3.1 Effect of Cell Detachment Method on Aptamer Binding

As described in Material and Methods section, the method used for detachment of A549 cells from cell culture dish was incubation of cells with 0.02 % EDTA solution. Binding of aptamers is dependent of well-preserved cell surface, therefore we have tested three different approaches of cell detachment as described in publications for already selected aptamers (Hu et al., 2012; Zhao et al., 2009).

Control aptamer (CTRL_N34) with random sequence of 34 nucleotides flanked with the same forward and reverse primers as tested aptamers was used. For determination of binding each respective sequence, binding values of the control aptamer were subtracted.

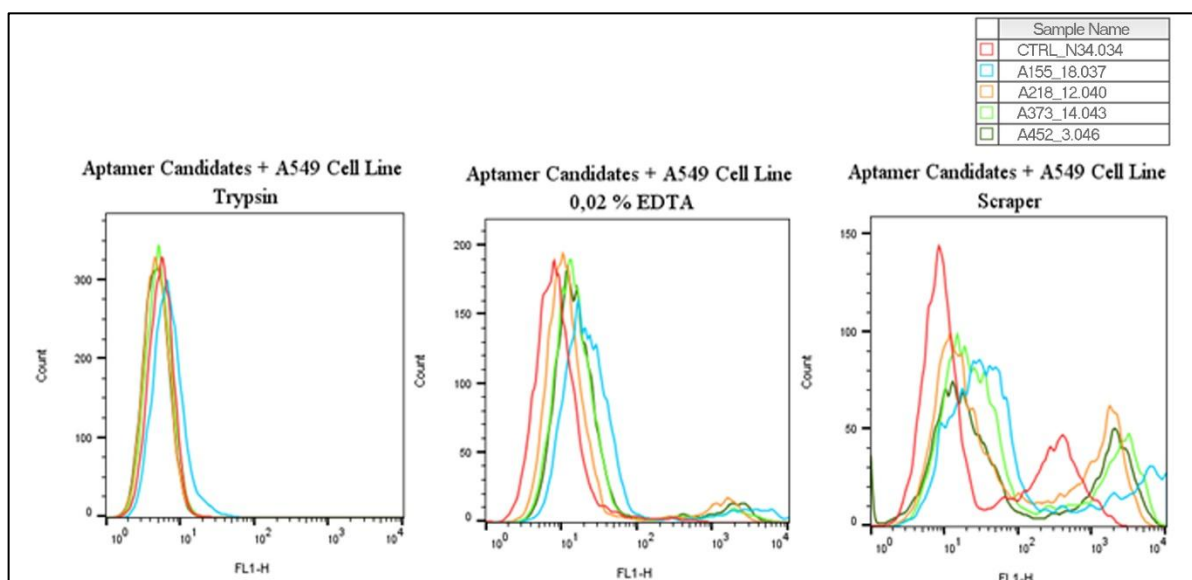


Figure 3.7: Representative flow cytometry profiles and quantitative analysis of binding selected aptamers after detachment with different approaches: 0.25 % Trypsin-EDTA, 0.02 % EDTA and scraper. Each selected aptamer is presented with different color: A155_18 (blue line), A218_18 (yellow line), A373_14 (green line), A452_3 (brown line). The control aptamer contained a random 34-nucleotide sequence (red line).

Table 3.6: % of fluorescent cells measured with flow cytometry, after incubation with selected aptamer candidates. Three different detachment approaches were used. Each measurement was repeated three times and % of control aptamer was subtracted.

	% of fluorescent cells		
	TRYPsin	EDTA	SCRAPER
A155_18	23.0	73.1	51.2
A218_12	2,6	36.2	36.6
A373_14	3.1	50.6	45.9
A452_3	9.3	53.5	42.4

3.3.2 Effect of Trypsinization on Aptamer Binding

In order to determine if the target of our aptamers is protein (or closely accompanied by proteins), we tested binding of aptamer candidate A155_18 with cells treated with different trypsin concentrations. As comparison we used cells detached only with EDTA as were detached for selection. With increasing trypsin concentration the 0.02 % EDTA was kept as constant.

We have observed the 95.2 % reduction in fluorescent signal already with the lowest trypsin concentration (Figure 3.8). With increasing concentration the % of fluorescent cells remained around 4 %: 3.25, 4.36, 3.43 and 3.55 respectively (Table 3.7).

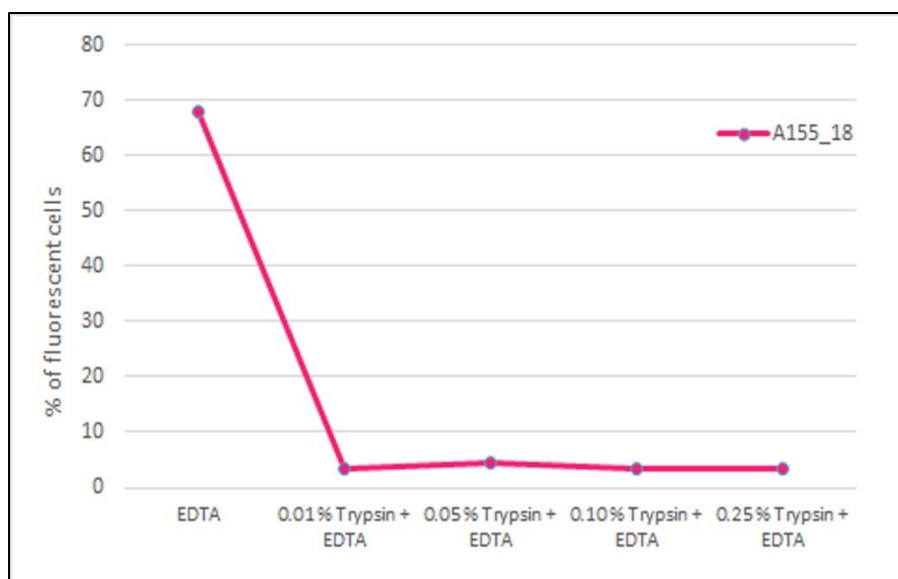


Figure 3.8: Effect of increased trypsin concentrations on aptamer binding. Aptamer with best binding properties from previous binding experiments was used (A155_18). Excessive decrease in binding (correlating with % of fluorescently labelled cells) was observed already with the lowest Trypsin concentration (0.01 %). The binding range stayed the same with increased concentrations (0.05, 0.10 and 0.25). Concentration of EDTA was kept constant at 0.02%.

Table 3.7: The percentage (%) of fluorescent cells measured with flow cytometry, after trypsin treatment of target cells. Aptamer candidate A155_18 was used. Four different trypsin concentrations with constant EDTA concentration were tested.

				% of fluorescent cells
0.02 % EDTA				67.96
0.02 % EDTA +	0.01	% of trypsin		3.25
	0.05			4.36
	0.10			3.43
	0.25			5.55

3.3.3 Temperature Effect on Aptamer Binding

As described in Material and Methods, selections and binding experiments were carried out at 25 °C. Physiological aptamer applications occur at 37 °C. We have tested also binding of aptamer candidate at 4 °C to test if slowed down cellular metabolism affects binding (Figure 3.9).

We have observed a temperature impact on labeling target A549 cells with all aptamer candidates. Candidate where binding temperature had the biggest impact on final count of fluorescently labeled cells was A218_12 with decrease from 33.5 % of labeled cells at 25 °C to 3.6 % at 4 °C and 2.9 % at 37 °C (Table 3.8).

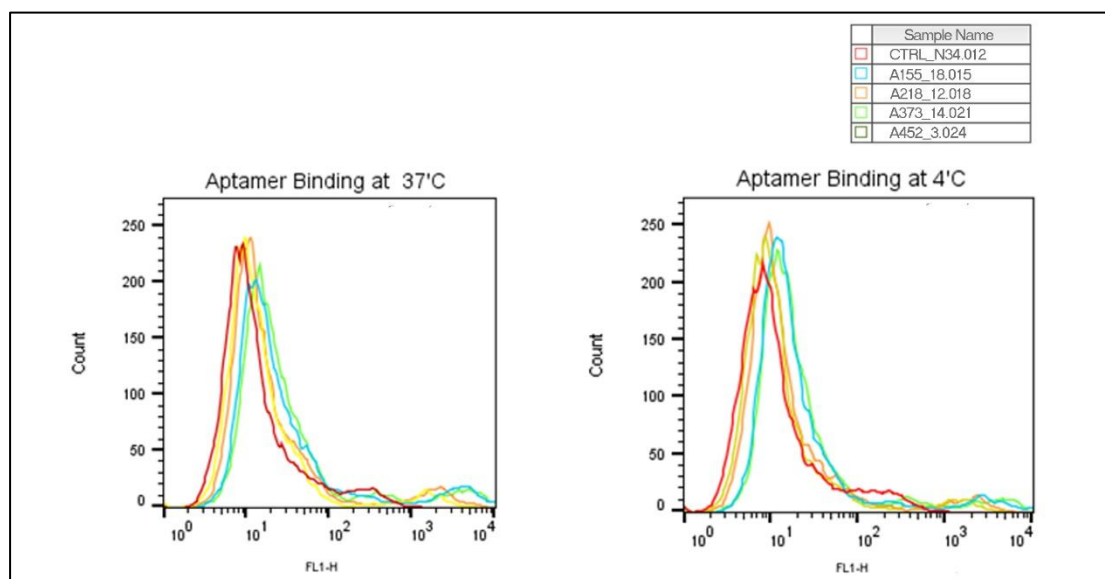


Figure 3.9: Representative flow cytometry profiles of binding selected aptamers at 37 °C and 4 °C. Each selected aptamer is presented with different colour: A155_18 (blue line), A218_18 (yellow line), A373_14 (green line), A452_3 (brown line). As control aptamer with random 34-nucleotide sequence was used (red line).

Table 3.8: % of fluorescent cells measured with flow cytometry, after incubation with selected aptamer candidates at three different temperatures: 4 °C, 37 °C and 25 °C. Each measurement was repeated three times and % of control aptamer was subtracted.

	% of fluorescent cells		
	4 °C	37 °C	25 °C
A155_18	28.9	20.0	64.1
A218_12	3.6	2.9	33.5
A373_14	11.0	8.8	52.8
A452_3	32.9	22.8	51.6

3.3.4 Passage number effect on aptamer binding

In order to test if target cell's surface epitope that is crucial for binding changes with sub-culturing cells to higher passages, we incubated aptamer candidates with target cell line passage 66 and passage 94 (Figure 3.10).

Decrease in binding on target cell line with higher passage was observed for aptamer candidates A218_12, A373_14 and A452_3. The binding for candidate A155_18 stayed the same (Table 3.9). That is showing on preserved binding epitope on target cells that is crucial for A155_18 binding.

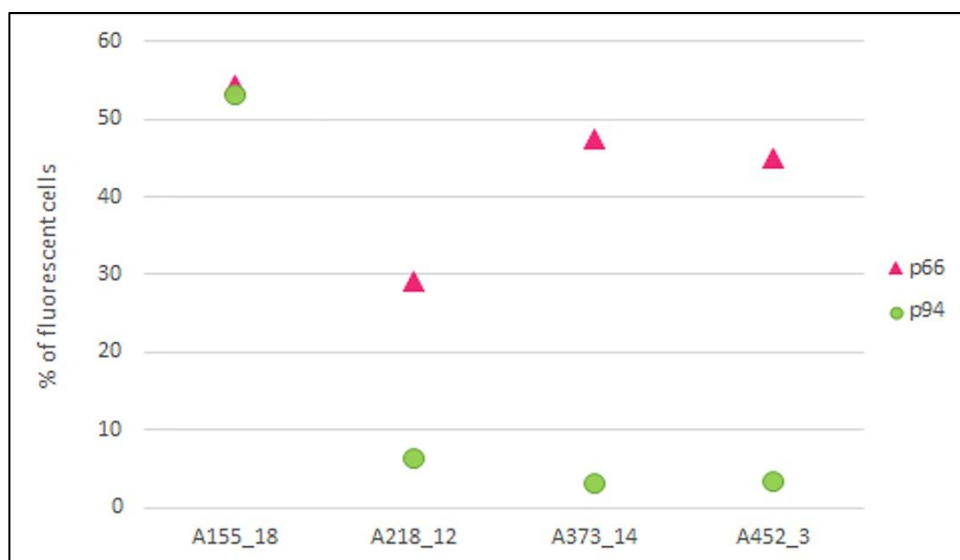


Figure 3.10: Values of fluorescent target cell line A549 of two different passages p66 (purple triangles) and p94 (green circle) when incubated with selected aptamer candidates A155_18, A218_12, A373_14 and A452_3. Binding of control random aptamer was subtracted to obtain final values

Table 3.9: Percentage of fluorescent cells measured with flow cytometry, after incubation of target cell line A549 of two different passages: p66 and p94 with selected aptamer candidates. Each measurement was repeated three times and % of control aptamer was subtracted.

	% of fluorescent cells	
	A549 p66	A549 p94
A155_18	54.3	52.0
A218_12	29.1	6.4
A373_14	47.5	3.2
A452_3	44.9	3.3

3.4 CD90 Protein Expression of the A549 Cell Line

Cell surface protein CD90 was chosen as specific marker for determination A549 cells as stem cells. The CD90 positive cells were used in final selection round for aptamer enrichment.

In order to compare CD90 protein expression and aptamer binding we compared the antibody and aptamer binding. For flow cytometry measurement Mouse IgG1 kappa monoclonal Isotype Control was used to determine background binding. The gate for further antibody was set to exclude non-specific background binding (Figure 3.11). Each measurement was repeated three times and an average CD90/Thy1 antibody expression was at 49.3 % of cells.

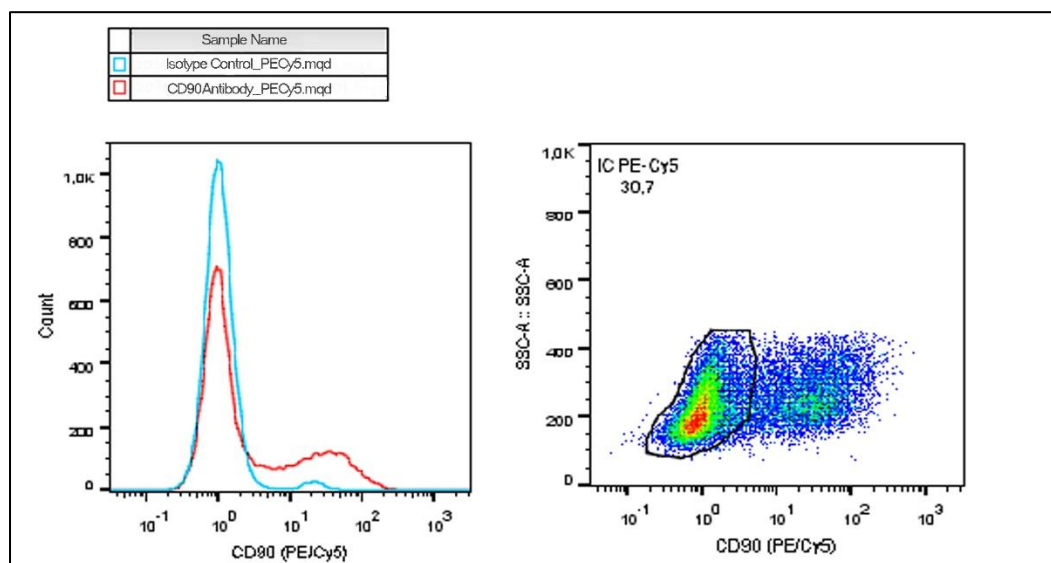


Figure 3.11: Representative flow cytometry measurement of protein CD90 presence on A549 cells' surface. As control Mouse IgG1 kappa monoclonal Isotype was used, to subtract non-specific binding (gated cell population on dot plot chart). Final average CD90 expression was shown at 49.3 % of total cell population.

3.4.1 Competitive Binding of Antibody CD90 and Aptamer A155_18

To perform a competitive binding experiment showed us that there is no correlation in binding of Antibody CD90 and selected aptamer A155_18 to target cell line A549 (Figure 3.12).

To exclude both options of occupying binding site we performed subsequent binding two ways. Incubation of cells first with antibody CD90 following incubation with aptamer A155_18 has shown a slight fluorescent increase (5.1 % and 6.3 % for aptamer and antibody respectively) in comparison with controls when cells were incubated with each tested ligand alone.

In second approach, we tested if aptamer A155_18 is occupying the binding site for Antibody CD90. Here we also observed only slight changes in fluorescence labeling in comparison with each ligand alone (0.3 % increase for aptamer and 9.3 % for antibody).

The major change in % of labeled cells was when we performed the binding experiment with antibody CD90 and aptamer A155_18 concurrently. Increase of fluorescent cells determined at FITC green filter was 12.9 % in comparison with increase for Antibody CD90 for only 2.8 %.

Those results are indicating that there is no competition between both of ligands, but increase of FITC fluorescence when incubated together can lead to conclusion that aptamer A155_18 is not only binding to A549 cells but also that there is an interaction between aptamer and antibody (Table 3.10).

Table 3.10: Competitive binding of aptamer A155_18 and antibody CD90 (AbCD90). % of labeled cells for each labeling approach was calculated based on three individual measurements. For each experiments cells were incubated with ligand alone (control) or in combination.

	% of fluorescent cells					
	APTAMER → ANTIBODY		ANTIBODY →APTAMER		ANTIBODY + APTAMER	
	A155_18	AbCD90	A155_18	AbCD90	A155_18	AbCD90
Alone (Control)	37.7	32.6	47.2	20.0	29.2	29.6
Combination	35.8	38.9	47.5	29.3	42.1	33.4

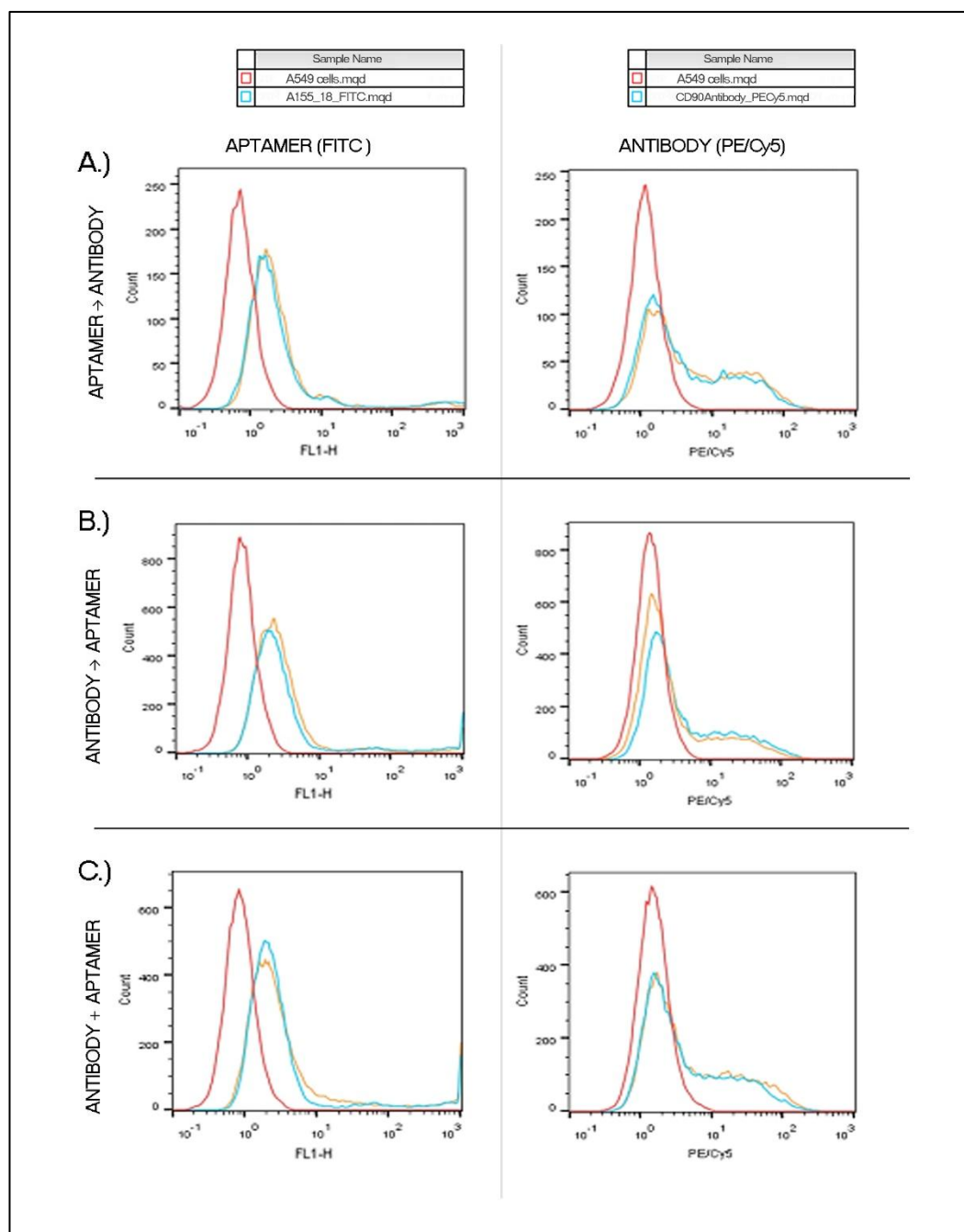


Figure 3.12: Representative flow cytometry measurement for competitive binding of aptamer A155_18 and antibody CD90. As each of the ligands carries different fluorophore: FITC and PE/Cy5, measurements were performed with both respective filters. Red line represents non-treated cell population, to exclude potential background fluorescence.

3.5 Comparison of Already Published Aptamer S6 and our Selected Best Binding Candidate

3.5.1 *In vitro* comparison using flow cytometry

When compared binding of both aptamers (S6 and A155_18), the flow cytometry results has shown that amount of fluorescent cells when incubated with aptamer S6 higher for 13.9 % (Figure 3.13). After three independent measurements for each, the obtained results were 57.5 % for aptamer S6 and 43.6 % for Aptamer A155_18 indicating that both aptamers are in similar binding frame ratio (Table 3.11).

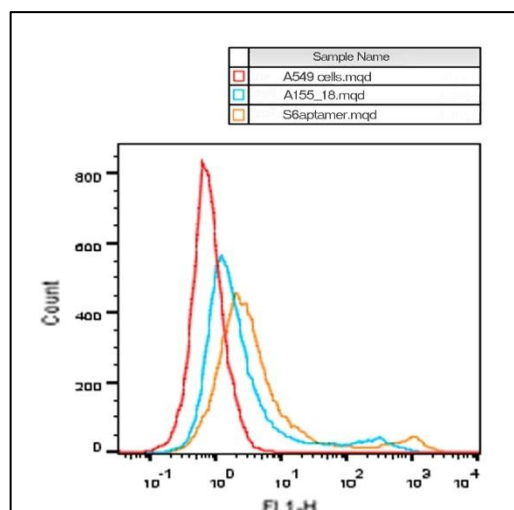


Figure 3.13: Representative flow cytometry comparison of binding aptamers A155_18 (blue line) and S6 (orange line). Non-treated cells are shown with red line.

Table 3.11: % of fluorescent cells measured with flow cytometry, after incubation of target cell line A549 with aptamers A155_18 and S6 respectively (p-value ≤ 0.001).

	% of fluorescent cells
A155_18	42.6
S6	57.5

3.5.2 Structure similarity using bioinformatics

Following *in vitro* experiments, we performed bioinformatics comparison of both sequences.

Dendrogram on Figure 3.14(A) illustrates clusters of aptamer sequence candidates where aptamer A155_18 and positive control S6 clustered together. Among the selected aptamer candidates, A155_18 and S6 are also the only two sharing two same motifs: GGTGG/CG and GCCAGT.

According to the UnaFold-predicted secondary structure, the motifs are placed into the comparable structural sequences between the loops Figure 3.14 (B). The possibility of cross-contamination was excluded as the 7th SELEX pool was sequenced before the positive control S6 was purchased.

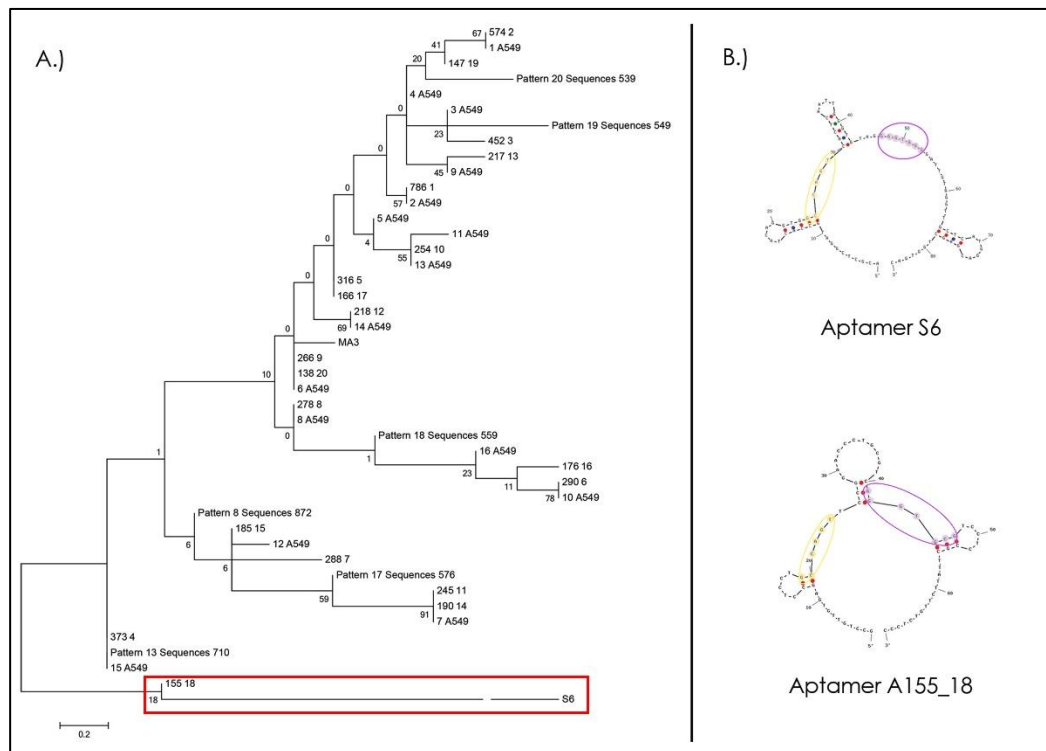


Figure 3.14: Bioinformatics comparison of two aptamers selected against the same target-A549 cell line. (A) Dendrogram representing evolutionary relations among selected sequences. Close relation of sequences aptamers A155_18 and S6 is presented by sharing branch. For dendrogram plotting MEGA program was used: sequences were aligned with ClustalW and dendrogram was plotted with the method of maximum likelihood and the substitution model Tamura-Nei (bootstrap 1 000). (B.) Comparison of secondary structures of A155_18 and S6 with lowest ΔG . Sharing motifs: are circled in violet (GGTGG/CG) and in yellow (GCCAGT). For the sequence folding simulation and ΔG calculation Unafold computer program (<http://eu.idtdna.com/unafold/Home/Index>) using energy rules: DNA, 25°C, 14.4 mM NaCl, 3.2 mM MgCl.

Chapter 4

Discussion and Conclusions

Aptamers, synthetic oligonucleotide molecules, are evolving from a random library whose diversity is comparable with naturally occurring sequences. Their binding ability comes from the tertiary structure in the presence of target molecules during SELEX. And up to date, those target molecules vary from small molecules up to whole organisms.

With the cell-based SELEX technique, new opportunities for the development of aptamers against cancer cells are arising, without knowing their crucial cell surface marker proteins. Using this approach, aptamers for multiple biomarkers can be developed and used complementary. And noteworthy, biomarkers recognized by aptamers selected using Cell-SELEX are in their native state on target cell surface, thus directly applicable for *in vivo* usage. This worthiness makes aptamers a promising tool for recognition of signature molecular profiles of cancer cells, defining diseases and development of bespoke, patient needs fulfilling treatment. Although more than twenty years of research and development of SELEX technique and aspiration towards improved aptamer pharmacokinetics qualities, their clinical applications are still being researched. Targeted cancer therapies are becoming the great focus of cancer research, utilizing their great precision and reduced side effects. In contrast to interfering the cell division as traditional chemotherapeutics acts, they interfere with the specific molecules crucial for tumor growth and progression. Up to now, the monoclonal antibodies are most successful and of the greatest importance for targeted cancer therapies. Despite their advantages, they are confronting with many disadvantages – high costs of development and production, utilizing large cultures of mammalian cells and also their large size, dependent limitation of penetration into tumor (J. W. Lee, Kim, & Heo, 2015; J. K. H. Liu, 2014). Aptamers, on the other hand, are produced with lower costs and providing more stable base for further modifications in order to achieve diagnostic and therapeutic purposes (Kong & Byun, 2013; Sun & Zu, 2015).

The needs for discovering new specific biomarkers are high and there is a need for a faster and more efficient selection process of aptamers. This dissertation provides a further support for the use of bioinformatics in order to promote selection process and to discover the key molecular interactions via specific biomarkers. Aptamers selected against specific cancer surface biomarkers hold a great potential in oncology for recognition of specific types of tumor cells in clinical samples. Latter is relevant for early diagnosis, prognosis and monitoring therapy responses, and in particular for targeting metastatic cells (Guo, Ziemer, Paul, & Wendel, 2008). This is also relevant for lung cancer treatment, one of the leading causes of death worldwide according to the latest WHO report (Ireson & Kelland, 2006; Kunii et al., 2011; K. Liu et al., 2013; Pei, Zhang, & Liu, 2014). Lung cancer is accounting for approximately 27 % of all cancer-related deaths (R. L. Siegel, Miller, & Jemal, 2016). Among main histological types of lung cancer, NSCLC has the highest incidence and is already locally advanced or metastasizes at the time of diagnosis (J. R. Molina et al., 2008). Therefore, the non-small lung cancer cells were chosen as model with the overall aim of development of DNA aptamers specific for non-small lung adenocarcinoma cells with cancer stem-like cells characteristics.

The aim of this study was using Cell-SELEX methodology for development of DNA aptamers, specific for non-small lung adenocarcinoma cells with cancer-stem like cell characteristics. The main *in vitro* approach, Cell-SELEX methodology, was further supported with *in silico* bioinformatics analysis of best binding aptamer sequences and validated by flow cytometry.

The lack of surrogate markers of antitumor activity that could provide an early indication of response to therapy and the problems in obtaining representative lung tumor specimens hinders the development of new therapeutic approaches for lung cancer patients (Berghmans et al., 2012; Eberhard, Giaccone, & Johnson, 2008). In blood and other body fluids, the early intravasating, so called CTC, are showing the prognostic and predictive capacity. Their determination, enumeration and characterization in easily accessed liquid biopsy represents a real-time inspection of the presence of metastizing cancer cells (Punnoose et al., 2012). Utilizing Cell-SELEX methodology, CTCs membrane represents an ideal structure for aptamer development (J. Huang, Wang, Xu, Huang, & Zhang, 2013). Some characteristics of lung cancer: aggressiveness, heterogeneity, drug resistance, recurrence and metastasis, could be attributable to the existence of a cancer stem cell (CSC) subpopulation with high capacity for self-renewal and differentiation. They have a capacity of invasion, metastasis, tumorigenicity and resistance to conventional therapy. And nonetheless, they are identified by variety of tumor-dependent cell markers. Lung cancer stem cells (LCSC) are CD44 and CD90 (Thy-1) positive subpopulation. It was shown that CD90+ lung adenocarcinoma A549 cells hold high proliferative and tumorigenic capacity.

The NSCLC cell line A549 was established in 1976 and has been very widely studied since then. They were initiated from a human alveolar cell carcinoma of a 58-year old Caucasian male. They are able to maintain type II alveolar epithelial cells properties at both, low and high passage levels that makes them an appropriate representative for lung cancer related studies (Gazdar, Girard, Lockwood, Lam, & Minna, 2010; Lieber, Smith, Szakal, Nelson-Rees, & Todaro, 1976).

Therefore, CD90 seems to be a promising new stemness marker for CSCs (Yan et al., 2013). As our first hypothesis of the study was stating, cell surface specific features of NSCLC cell line A549 are specific enough to promote selection of high selective aptamers using Cell-SELEX for those non-small lung cancer promoting cells, our last round of cell selection utilized the CD90+ sorted cell population.

To establish the truth of our hypothesis that CD90+ fraction of A549 cell population can be recognized by our selected aptamer, we have utilized the approach of concurrent labelling of cells with AntiCD90 antibody and A155_18 aptamer.

As shown on the Figure 3.2, this selective targeting approach was successful. The latter was further supported with labeling the A549 cell line with AntiCD90 antibody, where the ratio of labeled cells corresponded the ratio of aptamer labelled cells. We have shown that the binding site of aptamer does not overlap with CD90 protein, so therefore it can be concluded that there is a presence of another lung cancer CSC specific epitope.

Moreover, aptamers lost their binding efficiency after trypsin protease treatment, thereby suggesting their most likely target epitope could be another marker protein on cell surface. These results demonstrate that Cell-SELEX methodology is a powerful approach that holds a promising way of understanding, in our research, a lung cancer disease without prior knowledge of crucial molecular target. As the aptamers were selected against whole live cells in their native state, they could be further utilized for clinical assays. To prove their availability for *in vivo* experiments, we performed a test of binding at physiological conditions (37 °C), where the binding of our most promising aptamer remained unaltered.

The pharmacokinetics of aptamers is one of the crucial aspects of producing them as oligonucleotides with final clinical usage. Their therapeutic potency *in vivo* is affected by total plasma clearance – metabolic clearance, glomerular filtration and tissue (liver and spleen) uptake. Aptamers circulating half-lives increase as the clearance rate decreases. The most widely used modifications are conjugation to large moieties such as 40 kDa polyethylene glycol (PEG), cholesterol or diacyl lipids, addition of phosphorothiorate backbones, sugar modifications and 3'-terminus capping. Aptamers, currently undergoing clinical trials, are (with the exception of NU172) all modified for increased bio distribution and more effective site delivery (Gupta et al., 2017; Keefe, Pai, & Ellington, 2010). Therefore, for further usage of our selected aptamer as a potential drug bio conjugate or drug delivery vehicle, incorporation of such modifications would be an optimizing next step.

Starting with 10^{15} different sequences as input for the first selection round, even after introduction of counter target, in our case whole blood count, and further increased stringency of selection conditions, large amount of sequence data comes as an output after the last selection round. And in purpose to monitor the obtained oligonucleotide pool, the bioinformatics is used extensively. By using different sequencing technologies and bioinformatics approaches, the representative sequences are recognized easily.

Together with the desire to accelerate the discovery of the best aptamer and the rapid development of computational approaches, up to present different computational tools were developed (Alam et al., 2015; Bailey et al., 2015; Blind & Blank, 2015; Caroli et al., 2015; Dao

et al., 2016; Hoinka et al., 2014; Hoinka, Dao, et al., 2015; P. Jiang et al., 2014). As key features for analysis, the secondary structure (APTANI, AptaTrace, and AptaMotif) and sequence abundance in population (AptaCluster, FASTAptamer, and APTANI) are used.

In order to confirm the set hypothesis, that use of different bioinformatics tools will converge in the selection of the best binding aptamer sequences we have utilized three different bioinformatics approaches for characterizing our selected aptamers. Remarkably, despite their different way of analyzing the sequence data, the results of two of them were pointing out at sequence named A155_18 as the most promising one. It has shown good stability, motif uniqueness and high copy number in total final pool of sequences. It is important to note that *in silico* obtained results were supported with *in vitro* experiments. Our discovered sequence A155_18 has shown the best binding to target cell line A549 utilizing flow cytometry measurements. Therefore, the great potential of bioinformatics approach was shown and proven as an additional way of efficiently providing the best binding candidate for targeting yet unknown surface biomarkers.

In the last part of our work, the aptamer A155_18 was compared with already published aptamer S6 developed against the same target cell line.

Utilizing the bioinformatics comparison of aptamer sequences S6 and A155_18, we confirmed our hypothesis that the motifs crucial for target binding are preserved and recognized between sequences. Among a pool of narrowed-down sequences with the best binding characteristics based on bioinformatics scoring, only the sequences S6 and our A155_18 shared the same two motifs. And furthermore, their secondary structure and location of sharing motifs were comparable. Their relation was also proved by constructing the dendrogram where those two aptamers were sharing the same branch, located away from other sequences.

In conclusion, the results of the study have laid the foundation of different bioinformatics approaches on the way towards discovering crucial criteria for the best binding aptamer sequences. Our results provided a proof of concept for the use of bioinformatics as an important approach in already rapidly developing Cell-SELEX methodology towards recognition of novel cancer biomarkers.

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Publications Related to the Thesis

Journal Articles

Vidic, M., Motaln, H., Ulrich, H., Lah, T. T. (2015) Aptamer for imaging and therapeutic targeting of brain tumor glioblastoma. *Cytometry. Part A*: 87 (9) 806-816, doi: 10.1002/cyto.a.22715. [cobiss.si-id 3543631]

Vidic M., Smuc T., Janez N., Blank M., Acetto T., Mavri J., Nascimento I. C., Nery A. A., Ulrich H., Lah T. T. (2018) In silico selection approach to develop DNA aptamers for a stem-like cell subpopulation of non-small lung cancer adenocarcinoma cell line A549. *Radiology and Oncology*: 0 (0), pp., doi:10.2478/raon-2018-0014

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Vidic, M., Neves O., M., Breznik, B., Pillat, M. M., Glaser, T., Ulrich, H., LAH T., T.(2015) Interference of direct and indirect co-culture of glioblastoma and mesenchymal stem cells in expression levels of kininergic and purinergic components. *Abstract book*, 96-97, 142-143. [COBISS.SI-ID 3517263]

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Vidic, M., Motaln, H., Janež, N., Peterka, M., Ulrich, H., Lah T., T. (2016) Development of DNA aptamers specific for lung cancer stem-cells. *3rd International Symposium [on] Aptamers, 4th-5th April 2016, Oxford, United Kingdom*. str. 18. <http://libpubmedia.co.uk/aptamers-2016/documents-pdf/>. [COBISS.SI-ID 3925327]

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Biography

The author of this thesis, Mateja Vidic, was born on 10th of February 1989 in Ljubljana. She finished Primary school Zbora Odposlancev Kočevje and High school Kočevje. In 2007, she enrolled in Bachelor's study programme Biology and in 2010 in Master's study Programme Molecular Biology at Biotechnical Faculty, University of Ljubljana. She defended her master thesis, entitled Effect of HPV infection on responsiveness of squamous-cell carcinoma cell lines of hypopharynx to radiotherapy and chemotherapy in August 2013. The master thesis was performed under the supervision of Prof. Maja Čemažar.

In October 2013, she enrolled in the PhD program Nanosciences and Nanotechnologies at Jozef Stefan International Postgraduate School and in November 2014 started her PhD research as a young researcher at the Department of Genetic Toxicology and Cancer Biology at National Institute of Biology under supervision of Prof. Tamara Lah Turnšek and co-supervision of Prof. Henning Ulrich, Department of Biochemistry, Institute of Chemistry, University of São Paulo, Brazil. She spent two months of her PhD training in his laboratory. There, she performed Flow Cytometry and FACS experiments.

She presented her work at several international conferences and meetings, such as Glioma Invasion Aptamers 2016 and Computational and Molecular Biology Symposium 2017. The PhD candidate attended several workshops on the topics scientific writing and cancer biology.

