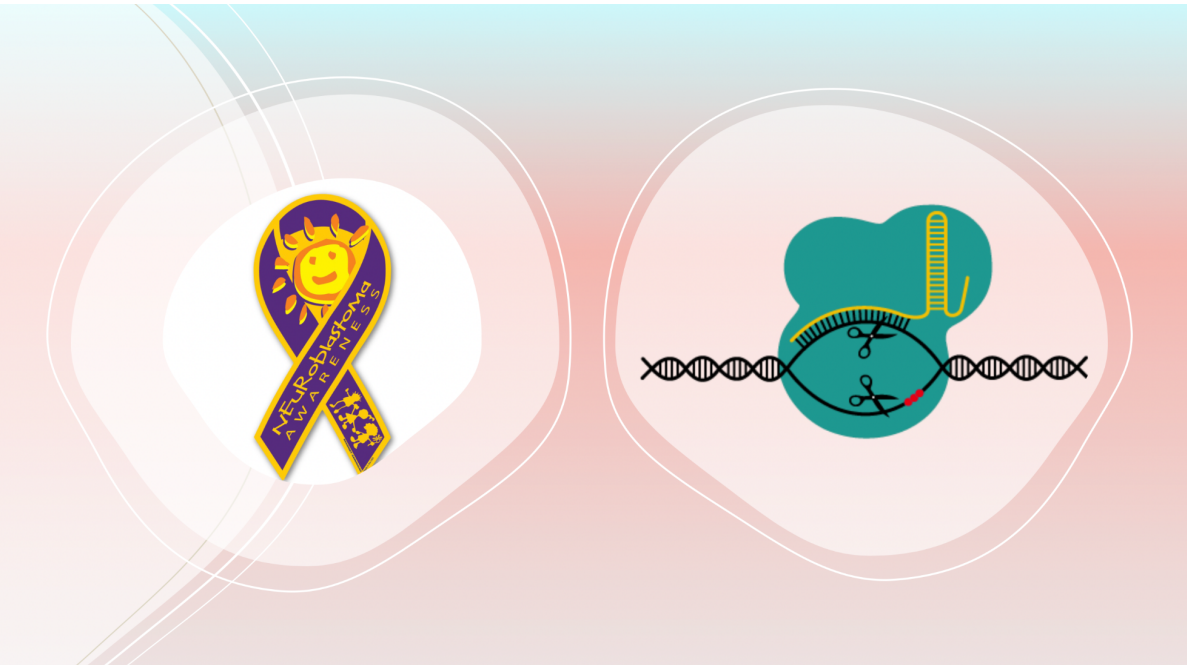

Exploring the Role of the *ATRX* Gene in High-Risk Neuroblastoma via Genome Engineering Techniques

Master's thesis in Biotechnology

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DEPARTMENT OF LIFE SCIENCE

CHALMERS UNIVERSITY OF TECHNOLOGY
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Cover: Utilizing genome editing techniques in Neuroblastoma.

Gothenburg, Sweden 2023

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Abstract

Neuroblastoma (NB) is a rare but aggressive pediatric cancer originating form the embryonic neural crest cells. Abnormal telomere maintenance has been implicated as a major cause of high-risk neuroblastoma, with a subset of tumors following the Alternative Lengthening of Telomere positive (ALT+) pathway. ALT+ tumors are difficult to treat due to their resistance to therapy. The exact cause of NB remains unclear, but recent research suggests genetic mutations as a possible cause, particularly mutations in the ATRX gene observed in ALT+ NB patients. This study focuses on understanding the role of the ATRX gene, in promoting ALT+ neuroblastoma. To investigate this, a dual approach was utilized, involving CRISPR/Cas9 technology for complete ATRX knockout and lentiviral shRNA silencing to reduce ATRX expression. The gene expression of ATRX has been successfully downregulated, but the successful knockout of ATRX could not be verified using CRISPR/Cas9 methods. Hence, additional validation is necessary to assess the impact of ATRX expression on ALT+ and to develop more effective strategies for addressing this subgroup of high-risk neuroblastoma (NB).

Keywords: Neuroblastoma, Lentiviral vector, ATRX, CRISPR/Cas9.

Acknowledgments

I would like to express my sincere gratitude to my supervisor, Dr. Tanmoy Mondal, for the patient guidance and support throughout the duration of my project. I am truly thankful for always keeping your door open and for giving me confidence when I needed it, especially during moments of disappointment.

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Lastly, I want to express my deepest gratitude to my family, with a special mention to my loving husband and daughter for their unwavering support and understanding. I am truly fortunate to have you in my life and without you, I could not have done this journey.

Setareh Jafargholizadeh, Gothenburg, July 2023

Contents

List of Acronyms

Below is the list of acronyms that have been used throughout this thesis:

Neuroblastoma (NB)
Alternative Lengthening of Telomere positive (ALT+)
alpha-thalassemia X-linked syndrome protein (ATRX)
trunk neural crest cells (tNCCs)
guide RNA (gRNA)
human embryonic stem cell (hESC)
Clustered regularly interspaced short palindromic repeats (CRISPR)
sequence-specific nucleases (SSNs)
double-stranded breaks (DSBs)
homology-directed repair (HDR)
nonhomologous end-joining (NHEJ)
protospacer adjacent motif (PAM)
RNA interference (RNAi)
small interfering RNA (siRNA)
short hairpin RNA (shRNA)
adeno-associated viruses (AAV)
Dulbecco's modified Eagle's medium (DMEM)
Fetal Bovine Serum (FBS)
Immunofluorescence (IF)
Phosphate Buffered Saline (PBS)
Phosphate Buffered Saline with Tween™ 20 (PBS-T)
Bovine Serum Albumin (BSA)
Tracking of Indels by DEcomposition (TIDE)
Single Nucleotide Polymorphisms (SNPs)

List of Acronyms

Below is the list of acronyms that have been used throughout this thesis:

Insertions or deletions (indels)
Puromycin resistance gene (PuroR)
4',6-diamidino-2-phenylindole (DAPI)
Green fluorescent protein (GFP)

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Below is the list of tables that have been used throughout this thesis:

1
Introduction

This chapter provides a short introduction to the thesis project, starting with an overview of Neuroblastoma, followed by an explanation of the techniques employed throughout this study. The chapter also outlines the project's aim, clarification of the issue being investigated, the study's limitations, and ethical concerns.

1.1 Background

Neuroblastoma (NB) is a rare but aggressive pediatric cancer that derives from neural crest cells, which are embryonic cells. Neural crest cells play an essential role in the formation of the nervous system and other tissues. They can differentiate into various cell types, including neurons, glial cells, and melanocytes (Gilbert 2000). In neuroblastoma, tumors arise as a result of the improper differentiation of trunk neural crest cells (tNCCs) which form the sympatho-adrenergic. One of the major causes of high-risk neuroblastoma is believed to be the abnormal maintenance of telomeres (Hertwig et al., 2016). A subset of high-risk neuroblastoma tumors, called Alternative Lengthening of Telomere positive (ALT+), maintains their telomeres in a telomerase enzyme-independent manner (Akter & Kamijo, 2021). These tumors are difficult to treat, often exhibiting therapy resistance and frequent relapse (Ackermann et al. 2018; Hartlieb et al. 2021). Although the exact cause of NB is not well understood, research suggests that genetic mutations and alterations in the expression of certain genes may play a role in the development of the disease (Brodeur 2003; Maris 2010). Lovejoy et al. found that mutations in the ATRX gene, which encodes a chromatin remodeling protein, are frequently observed in ALT+ NB patients (Lovejoy et al. 2012). Since altering the expression of ATRX can affect ALT+ NB patients, I set out to examine how ATRX mutation promotes ALT in NB. To this end, I performed a dual approach and used (i) the CRISPR/Cas9 technology to generate complete ATRX knockout, and (ii) lentiviral shRNA silencing to reduce ATRX expression. Additional details on the utilization of these two techniques for inducing mutations in ATRX to develop an ALT+ NB model will be presented in the upcoming chapter.

1.2 Aim

The aim of the current project was to generate an ALT+ neuroblastoma (NB) model, providing additional evidence of the significant role of ATRX in neuroblastoma. This was achieved by introducing targeted mutations in ATRX using the CRISPR-Cas9 system in human embryonic stem cells (hESCs) and employing lentiviral shRNA plasmids for further investigation.

1.3 Limitations

There are some limitations to the proposed research on creating an ALT+ NB model and they should be considered when interpreting the study results. First, the differentiation of cells in vitro may not perfectly simulate the in vivo environment. Therefore, validation of results should be considered as future research whenever possible. Secondly, it is possible that the CRISPR-Cas9 system may not always introduce the mutations of interest, which could potentially impact the validity of the model (Uddin et al. 2020; Flora & Welcker, 2017). Thirdly, the study is focused on one potential source of high-risk NB, telomere maintenance through ATRX mutation, but it may not consider other potential causes or factors that may be contributing to the disease. For instance, it has been documented in some studies that in addition to the ATRX deletion, amplification of MYCN and TERT rearrangement can affect telomerase activity in neuroblastoma (Hartlieb et al. 2021). Hence, while this study provides valuable insights into the mechanisms underlying high-risk NB, it is crucial to recognize that genetic mutations and epigenetic modifications are among other factors that may also play a role in the development of the disease.

1.4 Clarification of the issue under investigation

Gaining insight into the cells from an Alternative Lengthening of Telomere (ALT) perspective has the potential to uncover new developmental knowledge about ALT+ NB, which is yet to be explored. This understanding is expected to help develop more effective strategies to combat high-risk neuroblastoma. Considering the above-mentioned biological context, we aim to decipher the following hypothesis:

1. The use of the CRISPR/Cas9 system can lead to alterations in ATRX, resulting in a complete ATRX knockout.
2. Employing lentiviral shRNA to suppress ATRX expression can lead to alterations in ATRX.

1.5 Ethical concerns

Generally, the use of embryonic stem cells raises ethical concerns. Pluripotent stem cell lines can be created from the inner cell mass of the 5- to 7-day-old blastocysts. However, the ethical and political implications of human embryonic stem cell (hESC) research are controversial due to the fact that it necessitates the destruction of human embryos. Some people believe that an embryo is a person and that deriving embryonic stem cells from it is the same as murder. Others believe that the early embryo deserves special respect as a potential human being but that it is acceptable to use it for certain types of research provided there is good scientific justification, careful oversight, and informed consent from the donor. But in general, opposition to stem cell research is not monolithic (Lo & Parham, 2010). In the current project, we have taken ethical considerations into account by utilizing commercial human embryonic stem cells (hESC).

2

Theory

This chapter provides a concise theoretical background on the fundamental concepts and methods that form the basis of this study, aiming to enhance comprehension of the experimental findings. With a focus on the project's objectives, a brief review of Neuroblastoma is presented, followed by an explanation of the lentivirus shRNA knockdown vector system and CRISPR-Cas9 system, along with an exploration of their potential applications in gene editing for various biological and therapeutic objectives. Additionally, the mechanisms behind the lentivirus shRNA knockdown vector system and established methods for achieving gene knockdown using this tool are thoroughly reviewed.

2.1 Neuroblastoma

Neuroblastoma is a cancer that originates from early nerve cells, commonly found in embryos or fetuses. The term "neuro" refers to nerves, while "blastoma" denotes cancer that develops from immature cells (Neuroblastoma Tumors, n.d.). This cancer affects infants and young children (Brodeur, 2003; Urayama et al., 2007).

The Children's Oncology Group categorizes patients into low-, intermediate-, or high-risk groups depending on age at diagnosis, INSS stage, tumor histopathology, DNA index, and MYCN amplification status (“Neuroblastoma: Biology, Prognosis, and Treatment,” 2010). This study primarily focuses on high-risk neuroblastoma. The reported results indicate that while high-risk neuroblastoma exhibits notable responsiveness to chemotherapy, the long-term survival rates remain low, with only 30% to 45% of patients achieving complete response to multimodal therapy (Matthay et al., 1999). Therefore, leveraging the growing understanding of neuroblastoma tumor biology is crucial for developing innovative therapies targeted at high-risk neuroblastoma.

2.1.1 ATRX (Alpha Thalassemia/mental Retardation X-linked syndrome.)

ATRX, located on the X‑chromosome, is a gene responsible for encoding an ATP-dependent helicase belonging to the SWI/SNF family of chromatin remodelers. ATRX collaborates with DAXX to form a chromatin remodeling complex that facilitates the replication-independent deposition of the histone variant H3.3 at pericentromeric and telomeric regions (Lewis et al. 2010). Various types of cancers can be caused by ATRX mutation in children and adults. Neuroblastoma is the most commonly observed childhood extracranial tumor, with an incidence rate of 10.2 cases per million individuals under the age of 15 (Gerven et al. 2022; Mahapatra & Challagundla, 2023). In neuroblastoma, both ATRX multiexon deletions and point mutations have been reported, and these mutations are closely associated with ALT development (Ackermann et al. 2018; Brunner et al. 2016). Notably, these mutations are closely linked to the development of ALT, a telomere maintenance mechanism based on homologous recombination, and it is detected in 5–15% of cancer cases (Hoang & O'Sullivan, 2020).

2.1.2 Alternative lengthening of telomeres (ALT)

Telomeres function as protective caps at the ends of chromosomes. A telomere structure consists of five steps as depicted in Figure 1: a) Vertebrate telomeres consist of repetitive DNA sequences, forming double-stranded apart from the terminus which are made up of only the TTAGGG strand. b) This unique structure allows for t-loop formation. c) The shelterin complex, consisting of six subunits, binds to telomeric DNA. d) Two shelterin proteins (TRF1, and TRF2) directly bind to double-stranded telomeric DNA, while one (POT1) binds single-stranded telomeric DNA directly. e) Additionally, POT1, TPP1, and TIN2, along with RAP1, indirectly engage with double-stranded telomeric DNA through their interactions with other shelterin proteins (Cesare & Reddel, 2010).

When telomeres reach a critically short length, normal cells lose their ability to divide and eventually die. In contrast, cancer cells have the ability to bypass this limitation, and having longer telomeres allows them to continuously divide, potentially leading to immortalization (Reddel, 2000). The results indicated that approximately 85% of all human cancers avoid telomere shortening by increasing telomerase activity (Shay & Bacchetti, 1997). Within the remaining 15%, including those with unfavorable outcomes, most are able to maintain their telomere lengths by the mechanism called Alternative Lengthening of Telomeres (ALT). ALT depends on homologous recombination and is a critical target for cancer therapy (Cesare & Reddel, 2010). Telomere maintenance has been identified as a significant factor in high-risk NB (Ackermann & Fischer, 2019). Among high-risk NB subgroups, there is a category known as Alternative Lengthening of Telomere positive (ALT+), where telomeres are maintained independently of the Telomerase enzyme (MacKenzie et al., 2021). Although understanding the exact mechanisms of ALT has been challenging, recent progress has led to the identification of essential genes involved in ALT.

In a study involving 208 pretreatment NB patients, it was observed that patients with ATRX mutation are frequently ALT+ (Ackermann et al. 2018). However, the mechanism through which ATRX mutation promotes ALT in NB remains unclear.

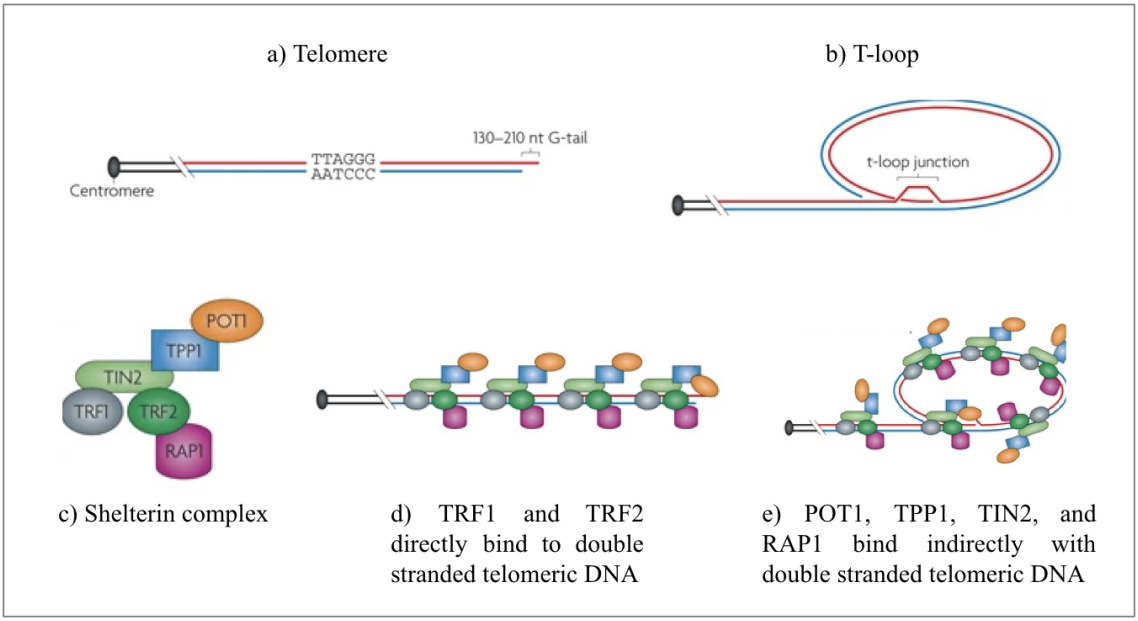


Figure 1: The structure of telomeres.

2.2 Genome engineering

Genome engineering is the process of making precise alterations to the genome, its epigenetic modifications, or its transcripts. This field has the potential to revolutionize basic science, biotechnology, and medicine, with efficient applications in eukaryotic and mammalian cells (Hsu et al., 2014). While this approach allows researchers to explore and understand genetic functions and interactions on a large scale, paving the way for advancements in various biological fields, it is still in its early stages of development (Carr & Church, 2009).

CRISPR-Cas9 and Lentiviral Vector-mediated RNA Interference are both considered genome engineering tools, each offering unique benefits. RNA interference has already demonstrated its efficacy in creating knockdown models for various diseases, while the introduction of CRISPR-Cas9 has become a valuable addition to the gene inactivation toolbox, enabling precise genome editing (Bellec et al., 2015). The following discussion will explore these two approaches in greater detail.

2.2.1 CRISPR-Cas9 system

Clustered regularly interspaced short palindromic repeats (CRISPR)-associated Cas9, known as designer nucleases, has emerged as a powerful genome-editing tool (Miyaoka et al. 2016). This revolutionary gene-editing technology has recently transformed the field of biology, offering significant potential for both experimental and therapeutic applications (Burgess 2013).

The CRISPR/Cas9 technology originates from type II CRISPR-Cas systems found in bacteria, which provide adaptive immunity against viruses and plasmids (Doudna & Charpentier, 2014). Shortly after encountering invasive genetic elements from bacteriophages or plasmids, a bacteria's immune system integrates short fragments of the foreign DNA into its chromosome. This process creates a genetic memory that allows the bacteria to recognize and eliminate the invaders quickly if they attack again. The CRISPR systems have been categorized into six different types (Makarova et al., 2015), each utilizing its unique set of Cas proteins along with crRNA for CRISPR interference (Wright et al., 2016). Unlike type I and type III systems that rely on large Cas protein complexes for cleaving foreign DNA (van der Oost et al., 2014), the type II system only requires one Cas protein to identify double-stranded DNA substrates and cleave each strand using separate nuclease domains (Gasiunas et al., 2012). Scientists were only able to carry out precise genetic modification after programmable sequence-specific nucleases (SSNs) became available (Sun et al. 2016). These SSNs cause double-stranded breaks (DSBs) at particular locations on the DNA's chromosomes. CRISPR has the ability to generate nicks or double-strand breaks within specific target regions, initiating two primary DNA repair pathways: homology-directed repair (HDR) and nonhomologous end-joining (NHEJ) (Miyaoka et al. 2016). While the HDR mechanism ensures precise DNA repair by utilizing homologous donor DNA, NHEJ represents a less accurate repair process, often resulting in various insertions and deletions, which contribute to genetic diversity (Miyaoka et al. 2016).

The CRISPR-associated protein Cas9 acts like molecular scissors, guided by a special RNA sequence (tracrRNA:crRNA), to find specific parts of the DNA. It forms base pairs with the DNA target, allowing Cas9 to cut the DNA at precise locations (Doudna & Charpentier, 2014). The successful recognition and cleavage of specific sites on DNA depend on the assembly of Cas9 with guide RNA (either a native combination of crRNA and tracrRNA or a synthetic sgRNA), forming an active DNA surveillance complex. The active CRISPR complex can be introduced into cells through various methods, including plasmids, in vitro transcribed RNA, precomplexed ribonucleoprotein, or viral vectors (Kouranova et al., 2016). The 20-nucleotide spacer sequence in the crRNA determines the specificity of the DNA target, while the tracrRNA plays a pivotal role in recruiting Cas9 (Jiang & Doudna, 2017).

Once Cas9 is bound to its guide RNA, the resulting complex becomes capable of searching for matching target DNA sites (Jiang et al., 2015). Recognizing the target involves two key elements: first, complementary base pairing between the 20-nucleotide spacer sequence and a corresponding sequence in the target DNA; second, the presence of a specific PAM (protospacer adjacent motif) sequence adjacent to the target site. The native PAM sequence is 5-NGG-3, where the letter "N" can represent any of the four DNA bases (Sternberg et al. 2014). When Cas9 identifies a target site with the appropriate PAM, it initiates a series of actions. It causes local melting of the DNA structure at the PAM-adjacent nucleation site, followed by the invasion of the RNA strand to create a hybrid structure between the RNA and DNA. This process results in the displacement of one DNA strand, forming what's called an R-loop, which extends from the PAM-proximal end to the PAM-distal end. Once the PAM is recognized and the RNA-DNA duplex is formed, the Cas9 enzyme becomes activated to cleave the DNA. For this purpose, Cas9 uses two nuclease domains. One is a conserved RuvC domain made up of three split RuvC motifs, while the other is an HNH domain situated in the middle of the protein. Each of these domains cleaves one strand of the target double-stranded DNA at a specific site located 3 nucleotides away from the NGG PAM sequence. This results in the production of a predominantly blunt-ended double-strand break (DSB), where only one strand of the DNA duplex is cut (Jiang & Doudna, 2017). In situations where sense and antisense sgRNAs are used to target opposite strands, Cas9 can make staggered cuts within the target DNA, leading to the creation of double nicks that induce a DSB (Ann Ran et al. 2013). Figure 2 illustrates the genome engineering process using the CRISPR-Cas9 system. In summary, a synthetic sgRNA directs Cas9 to a specific DNA sequence using a 20-nt guide RNA. Cas9 then creates a double-strand break (DSB) in the targeted DNA. Host repair mechanisms fix the DSB, primarily using error-prone nonhomologous end joining (NHEJ) or accurate homology-directed repair (HDR) if a template is provided. NHEJ often leads to random changes or disruptions in gene function. HDR can achieve controlled mutations through homologous recombination, allowing precise modifications like gene knock-ins, deletions, or corrections. The CRISPR-Cas9 system's RNA-guided targeting can be separated from its cutting function by altering the catalytic components within the HNH and RuvC nuclease domains. This modification transforms the system into a flexible tool for diverse applications beyond genome editing (Jiang & Doudna, 2017).

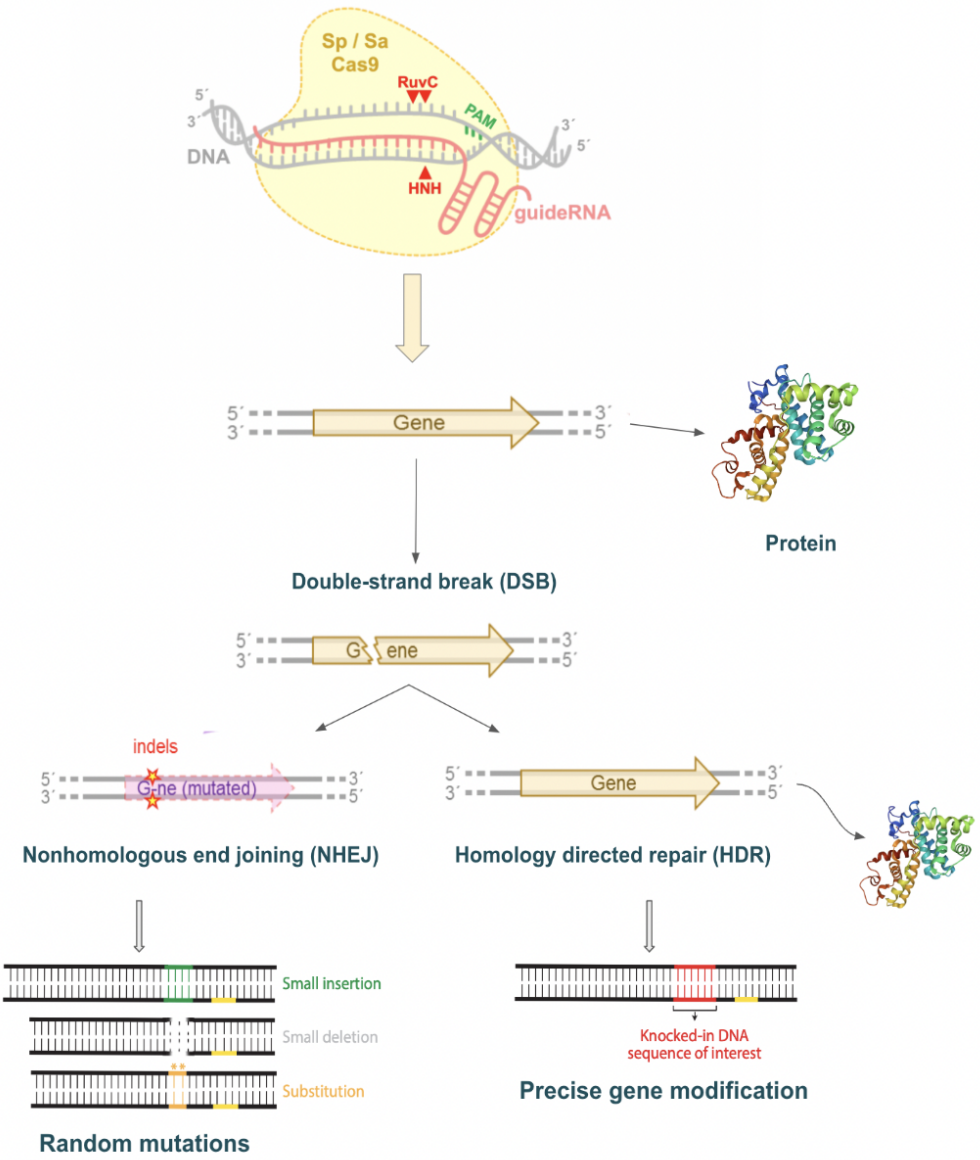


Figure 2: The process of genome engineering facilitated by the CRISPR-Cas9 system. Figure from Jafargholizadeh. Own work.

In recent years, CRISPR/Cas9 has found extensive applications in genetic modification and preclinical research on genetic disorders. However, a significant concern arises from the high frequency of off-target activity (≥50%), particularly for therapeutic and clinical purposes (Zhang et al., 2015). As an example, while the target specificity of Cas9 is generally thought to be determined by the guide sequence of the sgRNA, as well as the sequence of the PAM within the genome, there is still a possibility of off-target cleavage activity on DNA sequences with approximately three to five base pairs mismatched in the PAM-distal portion of the sgRNA-guiding sequence (Pattanayak et al., 2013; Zhang et al., 2015). Furthermore, different guide RNA structures have also been shown to affect cleavage of both on- and off-target sites in prior studies (Hsu et al., 2013).

Therefore, it becomes crucial to take into account the impact of off-target specificity within the CRISPR/Cas9 system. Such consideration will allow a more accurate interpretation of genome-editing data, thereby enhancing the utility of this technology in both basic and clinical applications.

2.2.2 Lentivirus shRNA Knockdown Vector

A Lentivirus shRNA Knockdown Vector is a specialized genetic tool used to decrease the expression of specific target genes within cells. This technology enables researchers to explore the consequences of gene knockdown, revealing the functions and roles of particular genes in various biological processes. In the subsequent paragraphs, I will provide a more comprehensive explanation of its functionality.

2.2.2.1 Short Hairpin RNA (shRNA)

RNA interference (RNAi) is a natural process that provides a precise and selective method for reducing the expression of specific target genes. This effect can be achieved through various techniques, including small interfering RNA (siRNA), short hairpin RNA (shRNA), and bi-functional shRNA (Rao et al. 2009). RNAi can be applied using two main approaches: chemically synthesized double-stranded small interfering RNA (siRNA) and vector-based short hairpin RNA (shRNA).

The effectiveness of RNAi was initially demonstrated using synthetic siRNA. Subsequently, it was observed that siRNA generated in vitro with the T7 RNA polymerase remained functional. Further studies revealed that active siRNA, structured in a hairpin, can be transcribed within cells from an RNA polymerase III promoter located on a plasmid construct (Miyagishi & Taira, 2002; Yu et al., 2002).

Shortly after discovering how cells use RNAi, scientists started using this powerful technique to learn about gene function. This included designing better ways for the efficient transportation of small interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs) into mammalian cells. The most straightforward RNAi approach involves directly delivering siRNA oligonucleotides to the cytoplasm. However, this method is limited to cells capable of transfection and is primarily employed in transient in vitro experiments. Consequently, introducing shRNA into mammalian cells through viral vector infection allows for the stable integration of shRNA, facilitating long-term reduction of the targeted gene's expression (Taxman et al. 2010). Figure 3 depicts the intracellular processing of shRNAs and miRNAs. Briefly, endogenous miRNAs, artificial miRNAs, and shRNAs are naturally synthesized as hairpin RNAs within the nucleus. After this formation, Drosha (a class 2 ribonuclease enzyme) cleave pre-miRNAs and artificial miRNAs with DGCR8, and together with shRNAs, transport them from the nucleus to the cytoplasm by Exportin 5 (Exp5). Within the cytoplasm, Dicer cleaves pre-miRNAs and shRNAs into active miRNAs and shRNAs. The presence of perfect binding sequences leads to the cleavage and degradation of target mRNAs, while imperfect binding, results in translational repression (Lambeth & Smith, 2013).

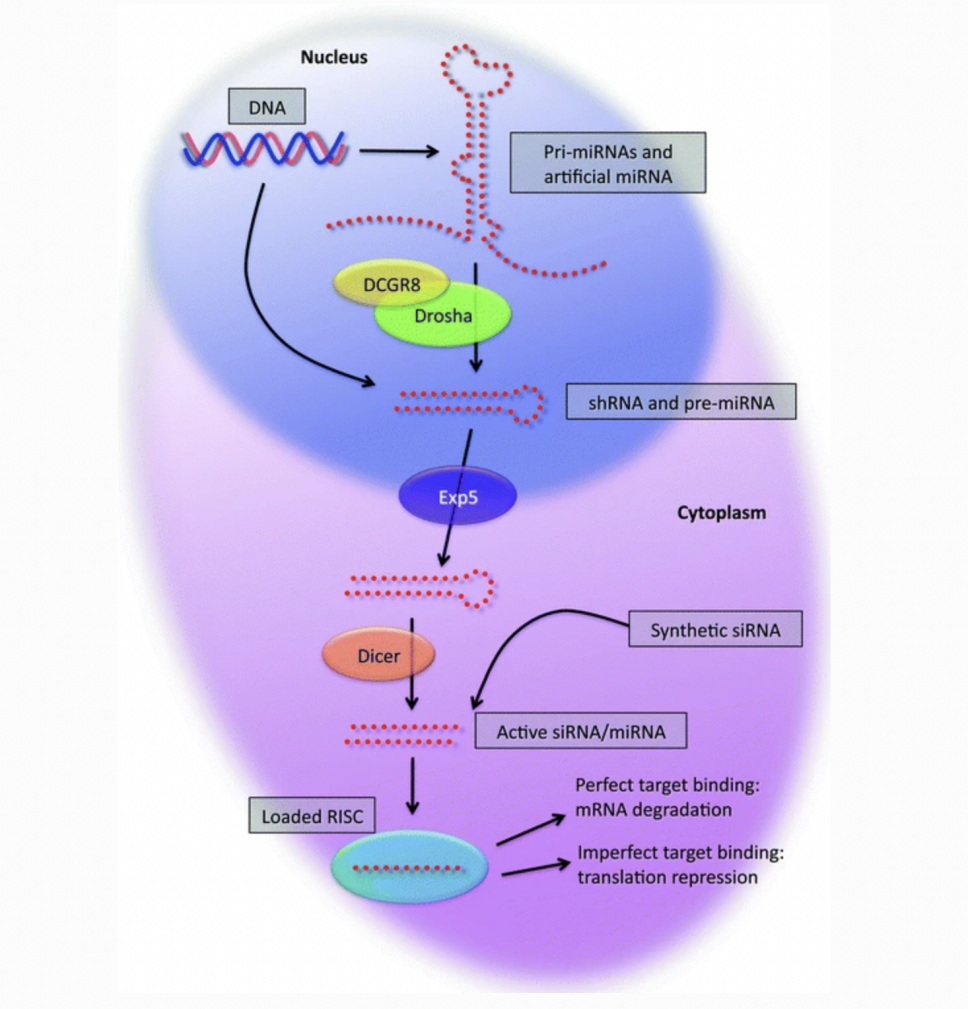


Figure 3: Processing of shRNAs and miRNAs within cells.

2.2.2.2 Lentiviral vectors

Various techniques exist for delivering siRNA and shRNA into cells. The selection of the method is dependent on the preference for transient or stable expression and the model system. Lentiviral-mediated transduction emerges as a favorable approach for introducing shRNA into both dividing and non-dividing cells (Singer & Verma, 2008). Moreover, this method is typically less toxic to cells compared to adenoviral-mediated transduction (Butt et al., 2022). Viral vector systems can be classified into two main categories: RNA viruses (like retroviruses and lentiviruses) that integrate into the host cell's chromosomes, and non-integrating DNA viruses (such as adenoviruses, adeno-associated viruses (AAV), and herpesviruses), which typically remain as episomes (Ghosh et al., 2020; Lambeth & Smith, 2013).

Retro- and lentiviruses possess a reverse transcriptase enzyme that facilitates their integration into host genomes as part of their natural lifecycle. By using recombinant viral vectors of these types, shRNA can be continuously expressed (Lambeth & Smith, 2013). Typically, non-replicating viruses are employed for transgene expression for safety considerations. This is achieved by introducing essential components of the viral genome into three or four separate plasmid vectors (Lambeth & Smith, 2013). This setup enables a single round of viral infection, followed by stable integration of the recombinant viral genome. As a result, the shRNA is consistently expressed without generating infectious virus particles (Lambeth & Smith, 2013). Figure 4 illustrates how genes are transferred by lentiviral vectors. Briefly, once the lentiviral vector infects a cell, like a hematopoietic stem cell, the RNA within the vector, which carries the therapeutic gene, is transcribed into DNA. This DNA then combines with the accessory protein Vpr, integrase enzyme, and matrix protein to create a preintegration complex. The localization sequences within these proteins facilitate the movement of the preintegration complex across the nuclear membrane. Once inside the nucleus, the DNA is integrated into the host genome using the integrase enzyme (Amado & Chen, 1999)

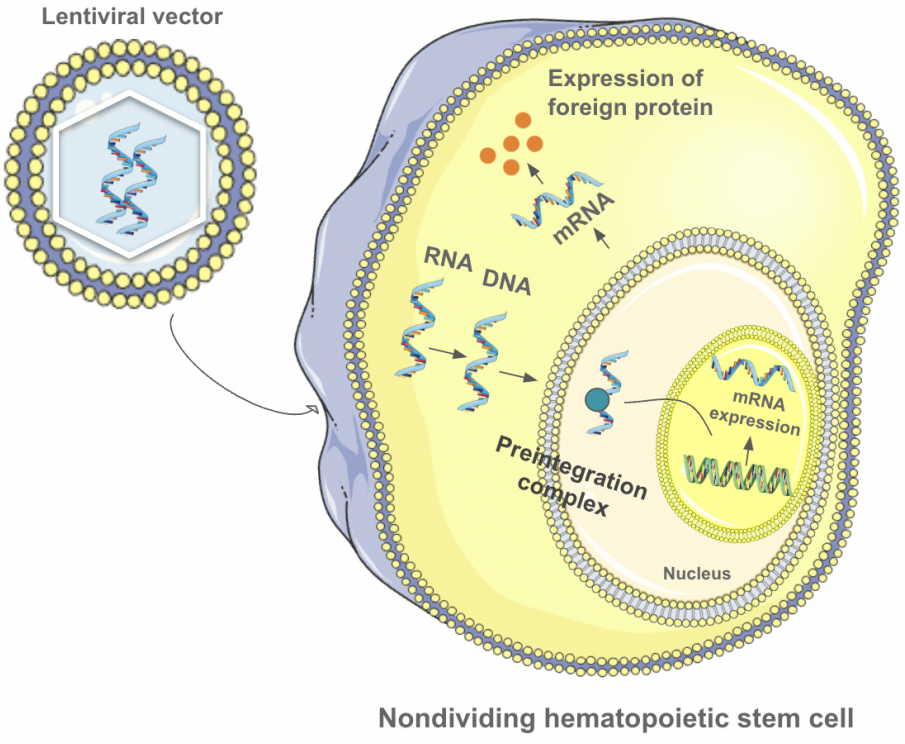


Figure 4: Lentiviral voyager. Transfer of genes by lentiviral vectors. Figure from Jafargholizadeh. Own work.

2.3 Embryonic stem cells (ESCs)

Embryonic stem (ES) cells derive from the early embryo and can remain pluripotent indefinitely in their primitive undifferentiated state (Pera et al., 2000). They have the potential to transform into a wide range of cell types within the human body (Pera et al., 2000). There are various types of stem cells, including embryonic stem cells and induced pluripotent stem cells (iPSCs) (Types of Stem Cell, n.d.) which in this study human embryonic stem cell was used. Due to two crucial properties, researchers find embryonic stem cells valuable for their studies. The first property is their ability for self-renewal, enabling them to continuously divide and replicate themselves. The second property is their differentiation capability, enabling them to differentiate into derivatives of all three embryonic germ layers; endoderm, ectoderm and mesoderm (Kleinsmith & Pierce, 1964; Rippon & Bishop, 2004). However, despite the vast potential of embryonic stem (ES) cells in medical research, human ES cell technology is still in its early stages, and many technical and ethical limitations must be addressed before any clinical application of ES cells.

3
Materials & Methods

This chapter provides a concise summary of the materials and methodologies employed in the study.

3.1 Cell Lines

3.1.1 Human embryonic stem cells (H9)

Human embryonic stem cells (H9) derived from embryos and they are adherent in culture. To grow human embryonic stem cells (H9), the manual protocol was followed.

3.1.2 Human Embryonic Kidney (HEK293)

Human Embryonic Kidney (HEK293) derived from human embryonic kidney tissue. HEK293 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Once the cells reached 80-90% confluency after 48 hours, they were detached from the flask by incubation with Trypsin for 3 minutes at 37°C. The prewarmed culture medium at 37°C was then added to stop Trypsin. Cells were seeded into two new 25T flasks with a subcultivation ratio of 1:3 and placed in humidified (37°C/5% CO2) incubator.

3.1.3 Human neuroblastoma cells (SHEP)

Human neuroblastoma cells (SHEP) derived from a neuroblastoma tumor. SHEP cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Once the cells reached 80-90% confluency after 72 hours, they were detached from the flask by incubation with Trypsin for 4 minutes at 37°C. The prewarmed culture medium at 37°C was then added to stop Trypsin. Cells were seeded into two new 25T flasks with a subcultivation ratio of 1:3 and placed in humidified (37°C/5% CO2) incubator.

3.2 Generating single Cell-Derived knockout clones in stem cells with CRISPR/Cas9

The protocol was consisted of several sequential sections. Initially, the knockout strategy was selected. Following that, gRNA target sites were identified, and vector cloning was performed. In the next step, gRNAs were introduced through lentiviral transduction. Subsequently, single-cell clones were isolated and expanded, and finally, knockout verification was conducted using western blot analysis and Sanger sequencing. All these steps were performed by Post-Doctoral Fellow Roshan Vaid, Ph.D., and Doctoral student Ketan Thombare, in the host laboratory.

3.3 Lentiviral ATRX Knockdown

As a control for inducible shRNA, we employed the pLKO.1-shRNA-Control plasmid, which had been supplied by the hosting laboratory. Additionally, the cloning vectors for ATRX (sh1 and sh2) were purchased from Sigma-Aldrich. Details regarding the utilized plasmid can be found in Appendix.

3.3.1 Plasmids

As depicted in Table 1, three different vectors were employed for Lentiviral shRNA. The control plasmid pLKO.1 contains only a vector backbone, which provides the essential components for plasmid replication within bacterial cells. As it does not have any shRNA sequences, it serves as a valuable reference to measure the efficiency of gene knockdown. Additionally, two ATRX shRNA plasmids with different shRNA sequences were utilized in generating the ATRX knockdown lentiviral expression vector. There is also an antibiotic-resistance gene on the control plasmid and ATRX_shRNA 1 & 2 plasmids.

shRNAs		
pLKO.1-puro Non-Target shRNA	SHC016, Sigma-Aldrich	Negative control
ATRX shRNA 1	TRCN0000013590, Sigma-Aldrich	CGACAGAAACTAACCCTGTAA
ATRX shRNA 2	TRCN0000013592, Sigma-Aldrich	CCGGTGGTGAACATAAGAAAT

Table 1: shRNAs-making plasmids.

3.3.2 Transfection

On Day 0, an almost confluent 75 cm² cell culture flask containing HEK293T cells (culture time < 6 weeks) was trypsinized with Trypsin/EDTA for approximately 3 minutes at 37 °C. The cells were then seeded in three 75 cm² flasks, with equal parts of 12.5 ml culture media in each flask. On Day 1, the culture media of cells to be transfected were replaced. (A): For each transfection (per flask), one 1.5 ml eppendorf tube was prepared. In each tube, 8.4 µg of the desired lentiviral expression plasmid, 6 µg of psPAX, and 3.6 µg of PMD2G plasmid were added.

Notably, psPAX and PMD2G were used as packaging plasmids. The volume was adjusted to 526 µl of plasmids with supplied H_2O. Then, 74 µl of the supplied 2M Calcium solution was added. (B): For each transfection (per flask), one 15 ml tube was prepared. In each tube, 600 µl of 2xHBS (supplied with the kit) was added. (A&B): The two solutions were mixed by carefully vortexing solution B while adding dropwise solution A. The transfection solution was incubated for 20 minutes at room temperature. Subsequently, 1.2 ml of each transfection solution was added to a 75 cm^2 flask, and the flasks were incubated overnight under cell culture conditions. On Day 2, the culture media of the HEK293T cells from Day 1 were replaced. On Day 3, the supernatant from each flask was poured into a 50 ml tube and allowed to stand on ice overnight. The flasks were then refilled with 12.5 ml of culture media. On Day 4, the supernatant from each flask was poured into the corresponding 50 ml tube from Day 3. The tubes were spun at 4100 x g for 15 minutes to remove cell debris. The supernatant containing the virus was frozen in working aliquots at -80°C for later use in the next step.

3.3.2 Transduction

A day prior to transduction, 1,500,000 SHEP cells were seeded into a T-25 (25 cm2) flask. Transduction was performed by seeding 200,000 SHEP cells into each well of the 6-well plate in 1.5 mL volume of DMEM complete media. These cells were added to the wells that already contained 0.5 mL of supernatant from the transfection step. To seed the cells, a batch of cells was prepared as follows: Dilute cells into a total volume of 10 mL of DMEM complete to have 200,000 cells into each well of the 6-well dish + 8 µg/mL polybrene (Since all the media in these wells was made with DMEM complete + 8 µg/mL polybrene, the final concentration of polybrene in each well should be 8 µg/mL). Finally, the cell suspension was mixed well by pipetting or inverting the tube. The cells were incubated with the virus for 48 hours. Then the media was replaced from the cells and 1.5 mL of DMEM complete was added containing 2 µg/mL of puromycin. After 24 hours of infection, cells were remained under selection to see how they respond to puromycin until all the mock-transfected cells died. Surviving cells were pooled and cultured for further analysis. The final results of the transduction part will be discussed in the next chapter.

Furthermore, in this step, an additional control was employed to assess the functionality of puromycin. One T-25 (25 cm2) flask of normal SHEP cells were cultured without puromycin.

3.4 Polymerase Chain Reaction (PCR)

PCR was used to amplify DNA from cultured cells (Using the QIAamp DNA Micro Kit). Cells were lysed in a 1.5 ml microcentrifuge tube with Buffer ATL, proteinase K, and Buffer AL, incubating at 56°C for 10 min, adding ethanol, and transferring the lysate to a MinElute column. The column was washed with Buffer AW1 and AW2 and then centrifuged to dry the membrane completely. After that, Buffer AE was added to the column for the elution of DNA. To ensure the complete elution of bound DNA, an equilibrated elution solution was utilized. Then SV Gel was used for DNA purification. The DNA was washed with membrane wash solution and then eluted with Nuclease-Free water. The resulting DNA was stored at 4°C.

3.5 Western blots

Western blots were employed in both techniques to identify the desired genes. Mutated H9 cells were used in the first technique to demonstrate the knockout result, while SHEP cells were used in the second technique to demonstrate the knockdown outcome. In each technique, to prepare whole cell lysates for western blotting, cells were resuspended with RIPA lysis buffer (150 mM NaCl, 1% NP- 40, 0.5% Sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 7.5, 1 mM DTT and protease inhibitor cocktail). After thorough mixing and 5 minutes of sonication, the samples were gently agitated at 14,000 × g at 4°C for 30 minutes. The resulting supernatants were carefully collected, and each sample was labeled in separate tubes according to the corresponding plasmid.

All samples were heated to 96C at the heat block for 5 min prior to loading. Lysed samples were combined with SDS-PAGE loading buffer (50 mM Tris-HCL pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT), and equal amounts and volumes of samples were resolved by SDS-PAGE. Proteins were separated through SDS-polyacrylamide gel electrophoresis on 15% gel and then transferred to a PVDF membrane by a wet electrophoretic transfer (Blotting) system. For this purpose, a standard transfer buffer was used to increase the hydrophobicity of proteins. The sandwich (sandwich western blot) was also organized from cathode to anode, respectively, with fiber pads, sponge, filter paper, polyacrylamide gel, membrane, filter paper, sponge, and fiber pads. To verify the successful transfer of proteins, a Ponceau S stain was applied before proceeding to blocking and incubation with primary antibodies (Table 2). The membrane was then blocked using a 5% PBS-T solution and subsequently incubated with the primary antibody overnight at 4°C. Details regarding the antibodies employed can be found in Table 2. The next day, the membrane was washed and incubated with secondary antibody (Anti-Rabbit IgG) at 1:5000 dilution in PBST–2% BSA, followed by another round of washing. The membrane was placed on a plastic surface with the protein side facing upward. The wrapped blot was then positioned, with the protein side up, in an X-ray film cassette for band visualization. Bands were quantified using Image Lab software.

Antibodies used in Western		
Anti-GAPDH rabbit polyclonal	5174, Cell Signaling Technology	Dilution 1:5000
Anti-ATRX rabbit polyclonal	ab97508, abcam	Dilution 1:1000
Anti-Rabbit IgG, HRP-linked secondary antibody	7074, Cell Signaling Technology	Dilution 1:5000

Table 2: The antibodies utilized in western blot.

3.6 Immunofluorescence

SHEP cells with ATRX_shRNA1 sequence (CGACAGAAACTAACCCTGTAA) and with ATRX_shRNA2 sequence (CCGGTGGTGAACATAAGAAAT) along with Control-sh (pLKO.1) were seeded onto a coverslip within a 24-well plate at a density of 200,000 cells. Cells were fixed using 4% formaldehyde for 10 minutes, followed by two times washes using PBS, and subsequently stored in PBS at 4°C until further use. Immunofluorescence (IF) was performed using a standard protocol. In brief, cells were permeabilized with 0.25% Triton X-100 for 10 minutes, followed by three washes with PBS-T. The cells were then blocked with a solution of 3% BSA in PBS-T for 30 minutes, and left with primary antibody overnight at 4°C. The next day, the IF process continued with 3 times PBS-T washes followed by incubation with respective secondary antibodies for 1 h at RT, and then 3 times PBS-T washes. Subsequently, the coverslips were dried and mounted onto a slide using DAPI. Images were captured utilizing a Confocal microscope. Details regarding the antibodies employed can be found in Table 3.

For imaging, coverslips were cleaned using an assay buffer and mounted in a chamber on the inverted microscope. The emitted light was filtered to capture either green or blue fluorescence. The assessment of the protein expression level will be discuss in next chapter.

Antibodies used in IF		
Anti-ATRX rabbit polyclonal	ab97508, abcam	Dilution 1:1000
Anti-Rabbit IgG (H+L), polyclonal secondary	A32732, Thermo Fisher Scientific	Dilution 1:800

Table 3: The antibody utilized in IF.

4

Results & Discussion

In this study, the objectives were divided into two main methods, and the results are presented accordingly. The first section outlines the results of ATRX knockout via CRISPR Cas-9, along with a brief discussion. Subsequently, the results of ATRX downregulation using shRNA plasmid are also illustrated and discussed.

4.1 ATRX expression verification

To focus on the role of ATRX in humans, we used human embryonic stem cells (hESCs). We generated ATRX knockout lines using CRISPR/Cas9, in addition to a lentiviral ATRX shRNA knockdown cell line. Subsequently, the ATRX expression levels were evaluated in both ATRX-shRNA1 and ATRX-shRNA2.

4.1.1 CRSPR/Cas9-Mediated Knockout of ATRX

Previous research on tumor cells has demonstrated a connection between Alternative Lengthening of Telomeres (ALT) and the lack of ATRX expression, either at the protein or gene level. In order to investigate whether the ATRX knockout in H9 cell lines activated the ALT mechanism, we utilized CRISPR-associated systems (Cas9). H9 clones carrying disrupted ATRX genes were verified through Sanger sequencing. As depicted in Figure 5, 10 clones were selected to be verified using Sanger sequencing . To analyze the mutations, TIDE (Tracking of Indels by DEcomposition) and Snapgene software were employed. According to Figure 5, among the 10 samples, ATRX-C5 (sample from the clone 5), ATRX-C10, ATRX-C11, and ATRX-C12 samples were selected as mutated samples (They are shown with red arrows in the Figure), while C7 were chosen as wild-type sample due to the absence of deletions or single nucleotide polymorphisms (SNPs).

In interpreting the graph, It's worth noting that the x-axis corresponds to nucleotide positions along the target sequence. Peaks in the graph reflect deviations from the wild-type sequence, and their variations provide an estimate of the presence of insertions or deletions (indels). Additionally, the graph is divided by a vertical red line (adjacent to the PAM sequence), with each side exhibiting distinct peaks. On the right side, steady peaks indicate an absence of mutations compared to the wild-type sequence. Conversely, on the left side, fluctuating peaks indicate potential indel events. Regarding the selected samples, it is evident that they exhibit more fluctuation compared to the other samples (ATRX-C5 in comparison with ATRX-C8).

The absence of ATRX protein expression in the knockout clones was verified through western blot techniques. Further details on this verification will be discussed later.

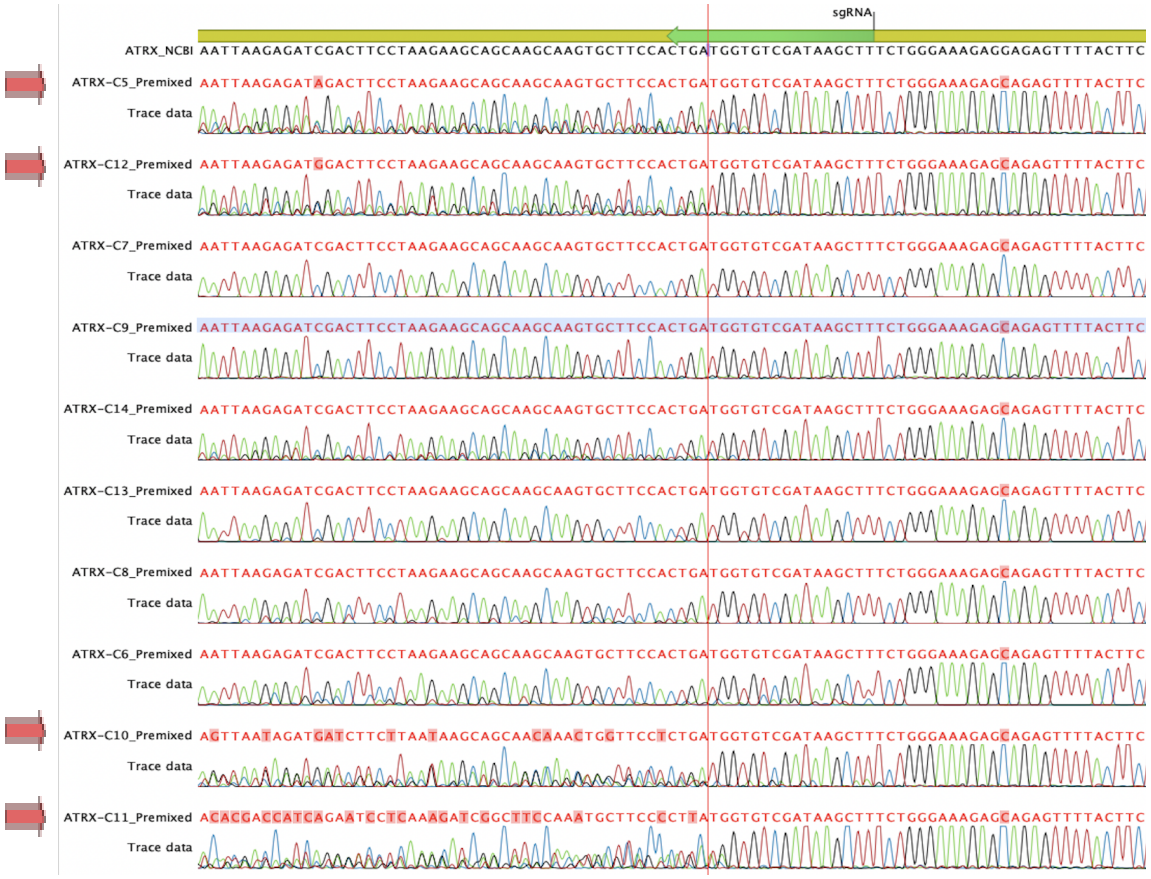


Figure 5: Analyze altered clones using TIDE software. The edited sequence, resulting from CRISPR/Cas9 activity, is distinct with a colored line, and for each nucleotide, there is a specific color (A: green, C: blue, T: red, G: black).

4.1.1.1 Western blot

Following the thawing of the chosen H9 mutated samples, the cells were seeded and subsequently harvested for ATRX expression verification using western blotting. As depicted in Figure 6, the western blot analysis did not confirm any reduction. Additionally, bands for the selected mutated samples (ATRX-C5 and ATRX-C12) were observed, indicating an unsuccessful ATRX knockout in human embryonic stem cells.

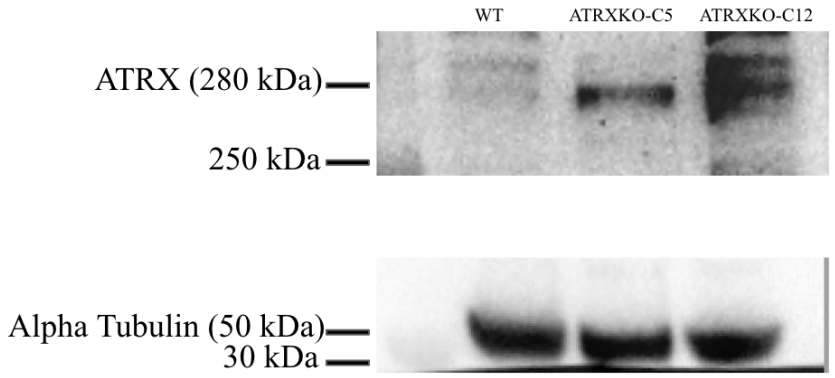


Figure 6: Western blot analysis of ATRX using CRISPR-Cas9 system. Alpha-Tubulin was used as a loading control.

In 2017, Bohaciakova et al. demonstrated an over 80% efficiency in the downregulation of p53 expression in hESCs through CRISPR-Cas9 (Bohaciakova et al., 2017). This emphasized the potential of the CRISPR-Cas9 system as a technique for suppressing the expression of target genes. Consequently, the initial phase of the current project centered on investigating the role of ATRX in humans using the CRISPR-Cas9 system.

The study could not confirm a significant reduction of ATRX protein through western blot analysis for the model cell line. To explore this further, various hypotheses will be discussed as potential reasons for the observed outcome. One possible reason could be related to the use of H9 cell lines which possess a normal female karyotype 46, XX. Given that ATRX is located on the X‑chromosome, the locus could carry two distinct alleles of a particular gene. In this scenario, H9 could be heterozygous for the ATRX gene and have a different genome sequence. Consequently, any mutation occurring in just one of the alleles could potentially be offset by the presence of the other allele. From a future perspective, employing H1 cells instead of H9 could prove beneficial. This is because H1 is a normal male karyotype 46, XY, with a single locus for the ATRX gene and a singular allele type.

The second possibility could be disrupted the function of the ATRX/DAXX complex. Recent research has revealed a consistent connection between ATRX mutations or the absence of ATRX and cell lines showing the ALT phenotype (Heaphy et al. 2011; Napier et al. 2015). Moreover, different cancers associated with the ALT phenotype are often linked to mutations in ATRX, DAXX, and/or H3.3 (Clynes et al. 2014). Hu et al. showed that ALT switches by inducing telomere-specific DNA damage, ATRX modification, and deletion of DAXX (Hu et al. 2016).

Similarly, the findings of Drané et al. indicated that DAXX and the chromatin remodeling factor ATRX (α-thalassemia/mental retardation syndrome protein) are specifically connected to the H3.3 deposition machinery and DAXX is a chaperone for H3.3 histones (Drané et al. 2010). Based on this observation, I hypothesized knocking out the DAXX gene meanwhile knocking out ATRX could be a potential way to stop ATRX expression in this model cell line.

The third possibility could be the presence of small deletions that may cause minor changes in the resulting protein. If deletions maintain the reading frame of the gene, they generally lead to only minor alterations in the resulting protein. Thus, I hypothesized that these small modifications might have limited functional consequences, possibly affecting the structure of the protein in a minor way.

In the case of the last possibility, since I didn't detect any homozygous knockout using Sanger sequencing, I hypothesized that the complete removal of the ATRX gene may lead to cell death. Considering all these scenarios, I shifted to a second strategy and employed lentiviral plasmids.

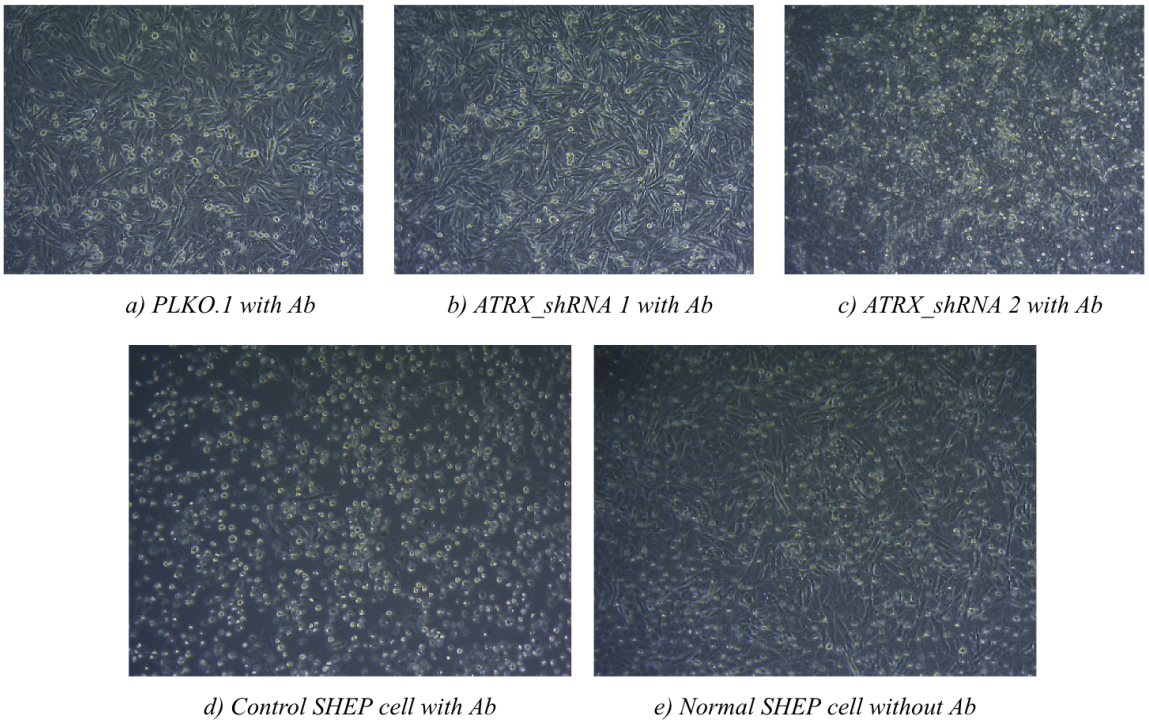
4.1.2 Lentiviral shRNA ATRX knockdown

To study the role and function of ATRX in neuroblastoma cells, we performed a lentiviral shRNA ATRX knockdown, which led to strongly reduced ATRX protein levels. For this purpose, lentiviral particles were exposed to SHEP cells. Following, ATRX expression in both ATRX-shRNA1 and ATRX-shRNA2 was analyzed using western blot and IF.

4.1.2.1 Post lentiviral transduction

Since the puromycin resistance gene (PuroR) encodes a protein that inactivates puromycin, cells with the PuroR gene become resistant to puromycin, allowing them to survive in the presence of the antibiotic. As depicted in Figure 7, cells that were treated with puromycin, including Control-sh (pLKO.1) and ATRX_shRNA1 & ATRX_shRNA2, were able to survive after the addition of puromycin (a,b,c). In contrast, SHEP cells without a resistance mechanism did not (d).

Additionally, normal SHEP cells which were cultivated without puromycin, also exhibited survival and It can confirm the functionality of puromycin (e).



a) PLKO.1 with Ab *b) ATRX_shRNA 1 with Ab* *c) ATRX_shRNA 2 with Ab*

d) Control SHEP cell with Ab *e) Normal SHEP cell without Ab*

Figure 7: Post-transduction conditions. Ab stands for Antibiotic resistance.

4.1.2.2 Immunofluorescence

The immunofluorescence analysis results, presented in Figure 8, Indicate a reduction in expression in SHEP-ATRX-sh1 and SHEP-ATRX-sh2 cells compared to SHEP-ATRX-Csh cells.

Anti-ATRX rabbit polyclonal (primary antibody) and Anti-Rabbit IgG (secondary antibody) were used for the Immunofluorescence. Moreover, the targeted protein was labeled with green fluorescent protein (GFP) to indicate gene expression and determine protein localization. The green spots in Figure 8 depict the expression of ATRX. Since SHEP-ATRX-Csh serves as the control to confirm diminishing expression, more green spots were detected in SHEP-ATRX-Csh compared to SHEP-ATRX-sh1 and SHEP-ATRX-sh2. Additionally, 4′,6-diamidino-2-phenylindole (DAPI) was used to outline the nuclei. DAPI is a blue-fluorescent DNA stain commonly used as a nuclear counterstain in fluorescence microscopy and staining. Each of the blue dots was detected as a nucleus. Subsequently, using ImageJ, cell numbers were counted by calculating the blue dots. As depicted in the Figure 9, the bar graph shows that the cell count in the SHEP-ATRX-Csh plasmid was higher than in the other plasmids. Additionally, SHEP-ATRX-sh2 exhibits a greater cell count compared to SHEP-ATRX-sh1.

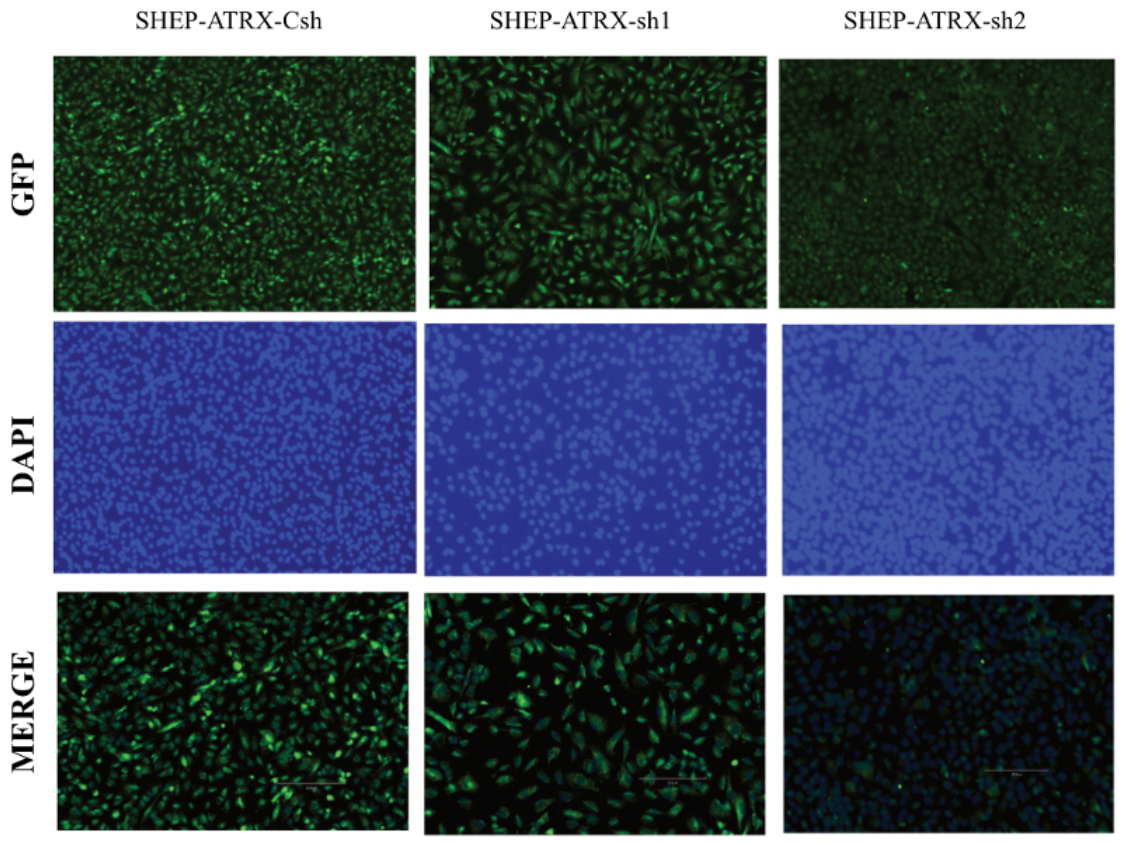


Figure 8: Immunofluorescence analysis of ATRX (scale bars = 300 µm). DAPI was used to outline the nuclear boundaries.

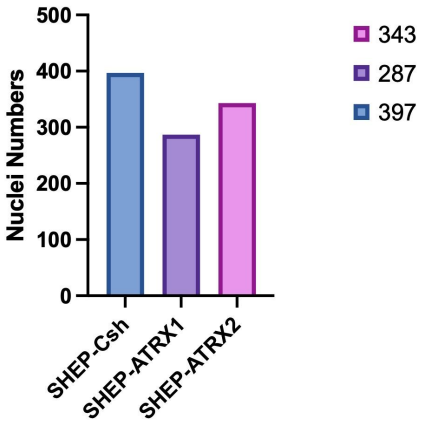


Figure 9: The number of nucleus in IF. DAPI was used to outline the nuclear boundaries.

4.1.2.3 Western blot

To verify the staining outcomes, we used a western blot using Anti-ATRX rabbit polyclonal antibody (primary antibody). The outcome of the western blot is illustrated in Figure 10. The ATRX protein is estimated to be around 280 kDa, and the observed band corresponds to the correct size of the ATRX protein. The results indicated low expression of the ATRX protein in SHEP_ATRX_sh1 and SHEP_ATRX_sh2 cells, validating the previous findings from immunofluorescence analysis. Furthermore, more expression was visible in SHEP-Csh cells. Moreover, as shown in Figure 10, measuring gene expression through a comparison of the intensity of alpha-tubulin as a loading control and the intensity of ATRX indicated a 3% downregulation for cells with the ATRX_sh1 plasmid and a 19% downregulation for SHEP cells with the ATRX_sh2 plasmid.

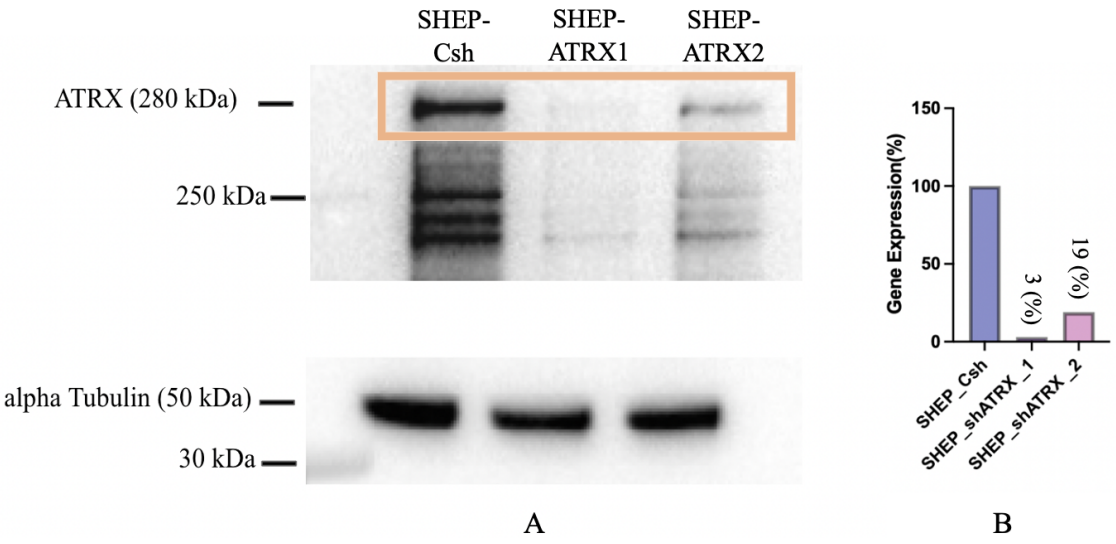


Figure 10: Western blot analysis of ATRX using lentiviral shRNA (A). ATRX expression (%) in SHEP-Csh, SHEP-ATRX1 and SHEP-ATRX2 cells (B).

Regarding to masure of ATRX expression in SHEP cells, gene expression levels were determined by comparing the ratio of the total band Vol. (Int) of ATRX in each specific plasmid, with the total band Vol. (Int) of alpha-tubulin to the ratio of the total band Vol. (Int) of ATRX in the control plasmid to the total band Vol. (Int) of alpha-tubulin. Alpha-tubulin was used as a loading control in Western blot analysis, as outlined in the Table 4.

Lane No.	Sample	ATRX/Alpha-TUBULIN	Gene expression
1	SHEP_Csh	0,501	100
2	SHEP_shATRX _1	0,015	3
3	SHEP_shATRX _2	0,095	19

Table 4: The ratio of the total band volume (Int) of ATRX to the total band volume (Int) of alpha-tubulin and the percentage of gene expression.

In the current study, considering the previously mentioned point and the pivotal properties of viral vectors, including safety, stability, and cell type specificity, we employed this approach to reduce the expression of the ATRX gene in SHEP cells (Song & Yang, 2010). In general, to focus on the role of ATRX in neuroblastoma patients, we used two distinct lentiviral ATRX shRNA vectors to silence the ATRX gene's expression within SHEP cells. The western blot results demonstrate downregulation of ATRX protein in SHEP cells when using both plasmids compared to the control, with a reduction of 3% for shRNA1 and 19% for shRNA2 as compared to the control (100%).

This outcome result is in agreement with priovius reports describing the sufficient ATRX knockdown with different plasmids and different cell line models (Hu et al. 2016). In summary, our findings from western blot and immunofluorescence (IF) confirmed the downregulation of ATRX expression in SHEP cells using lentiviral shRNA vectors. The western blot results demonstrated that the expression of ATRX in SHEP cells with the shRNA1 plasmid was lower than that with the shRNA2 plasmid. In comparison to the control, the results confirmed downregulation of ATRX gene expression with both shRNA1 and shRNA2 lentiviral plasmids. Moreover, the staining results indicated that both vectors worked successfully.

In 2019, Milenkovic et al. utilized both lentiviral shRNA to reduce TSPO expression and CRISPR/Cas9 technology to create complete TSPO knockout microglia cell lines (Milenkovic et al., 2019). However, the use of these two techniques to generate a cell model that can affect ATRX gene expression in human neuroblastoma and human embryonic stem cells has not been reported so far. Hence, in the future, employing ATRX shRNA1 and ATRX shRNA2 plasmids in human embryonic stem cells to diminish ATRX expression may represent one more step toward understanding how ATRX influences ALT in neuroblastoma disease.

5

Conclusion

To explore the role of ATRX in ALT in nurobeastma patiants , we adopted a dual approach: (i) employing lentiviral shRNA to diminish ATRX expression, and (ii) utilizing CRISPR/Cas9 technology to create a complete ATRX knockout. Our research indicated the supressing of ATRX expression up to 97% in human neuroblastoma cells, highlighting the efficacy of Lentiviral shRNA as a successful strategy to achieve this outcome. However, confirming the successful ATRX knockout through CRISPR/Cas9 systems in human embryonic cells proved challenging. While the achieved results showcase promising advancements in supressing of ATRX in human neuroblastoma cells through lentiviral shRNA, acknowledging the need for further improvement is essential. Specifically, stoping expresion of ATRX in human embryonic cells remain an ongoing area for development. Nevertheless, considering that this study marks the initial success in applying lentiviral shRNA in SHEP cells to achieve downregulation of ATRX, it can be a basic foundation for future studies to investigate ATRX expression in human embryonic cells.

6

Outlook

This chapter aims to provide ideas and direction for future studies.

As a first future perspective, employing H1 cells instead of H9 could prove beneficial. This is because H1 has a normal male karyotype (46, XY) with a single locus for the ATRX gene and a singular allele type.

Additionally, in the future, given our success in downregulating ATRX in the human neuroblastoma cell line, we can explore applying this approach to knock down ATRX in human embryonic stem cells.

Finally, since the main question of this project remains unanswered due to a lack of time for ALT measurement, there is potential for further investigation and answer how the knockout of ATRX affects ALT, in addition to considering its knockdown effects.

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