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Manuscript

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«Development of a cell therapy approach for diabetes by engineering tunable insulin production in β-cells»

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NORMATIVE REFERENCES

The dissertation was written according to the following standardized references:

- MEMCT 7.32-2001 – Provisions on scientific research. Rules for structure and preparation;
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DEFINITIONS

In the dissertation, the following terms are used with the corresponding definitions:

- **Pancreas** is a vital organ with both exocrine and endocrine functions, playing a crucial role in digestion and glucose metabolism. It is involved in various diseases, including diabetes and pancreatitis, and has been a subject of extensive research to understand its development, function, and associated pathologies;

- **β-cells** (beta cells) are specialized endocrine cells located within the pancreatic islets of Langerhans responsible for the production and release of insulin;

- **Insulin** is a crucial hormone primarily responsible for regulating blood glucose levels and plays a significant role in various physiological processes. It is secreted by the β-cells of the pancreas and works in conjunction with glucagon to maintain glucose homeostasis. Insulin's role extends beyond glucose regulation, impacting numerous cellular functions and influencing the development of chronic diseases;

- **CRISPR** (short for "clustered regularly interspaced short palindromic repeats") is a technology that research scientists use to selectively modify the DNA of living organisms. CRISPR was adapted for use in the laboratory from naturally occurring genome editing systems found in bacteria;

- **Cas9** is a nuclease and a part of CRISPR/Cas9 revolutionary genome-editing technology. Cas9 is guided by RNA, enables precise modifications to DNA, making it a powerful tool for gene editing across various organisms;

- **Guide RNA** (gRNA) is a specific RNA sequence that recognizes the target DNA region of interest and directs the Cas nuclease there for editing; the gRNA is made up of two parts: crispr RNA (crRNA), a 17-20 nucleotide sequence complementary to the target DNA, and a tracr RNA, which serves as a binding scaffold for the Cas nuclease;

- **Lentiviral vector** is a type of vector for delivering a sequence of interest and that is based on retrovirus which can infect both dividing and nondividing cells because their preintegration complex (virus "shell") can get through the intact membrane of the nucleus of the target cell;

- **HEK 293 cells** (Human Embryonic Kidney) is a cell line commonly used for biopharma and basic medical research and therapeutic solutions; generated in 1973, they are popular, in part because of their reliable growth in culture and for their propensity for transfection;

- **Plasmids** are extrachromosomal DNA elements found in bacteria that play a crucial role in genetic exchange and evolution. They are key vectors for horizontal gene transfer, carrying genes that can confer antibiotic resistance, virulence, and other traits beneficial for bacterial survival and adaptation;

- **VP64** transcriptional activator is a key component in CRISPR-based gene activation systems, which are used to modulate gene expression for research and therapeutic purposes. These systems utilize a deactivated Cas9 (dCas9) protein fused with VP64 to activate transcription of target genes;

- **KRAB domain** (Krüppel-associated box) is a highly conserved transcriptional repression domain found in a significant portion of zinc finger proteins across various species. It plays a crucial role in gene regulation, particularly in silencing transposable elements and influencing developmental and physiological processes;

- H1 embryonic stem cells is pivotal in regenerative medicine due to their ability to differentiate into various cell types. The line, in particular, has been extensively studied for its unique properties and potential applications;

- **CRISPR gene regulation** is a transformative approach in genetic engineering which allows precise control over gene expression. The technology leverages the CRISPR/Cas system to modulate transcriptional activity, offering insights into gene function and potential therapeutic applications;

- **Synthetic transcription factors** are engineered proteins designed to regulate gene expression with high specificity and efficiency. The molecules are constructed by combining DNA-binding domains with effector domains, allowing precise control over transcriptional activity.

NOTATIONS AND ABBREVIATIONS

DM	-	Diabetes mellitus
T1D	-	Diabetes type 1
WHO	-	World Health Organization
IDF	-	International Diabetes Federation
HEK	-	Human embryonic kidney
PBS	-	Phosphate buffered saline
GFP	-	Green fluorescent protein
Cas9	-	CRISPR associated protein 9
CRISPR	-	Clustered regularly interspaced short palindromic repeats
KRAB	-	Krüppel-associated box
ELISA	-	Enzyme-linked immunosorbent assay
RNA	-	Ribonucleic acid
DNA	-	Deoxyribonucleic acid
gRNA	-	Guide RNA
AAV	-	Adeno-Associated Viral
IAA	-	Insulin autoantibodies
ICA	-	Islet cells
Zn-T8A	-	Zinc transporter
GADA	-	Glutamate decarboxylase
IA-2A	-	Tyrosine phosphatase-like protein
DKA	-	Diabetic ketoacidosis
ESCs	-	Embryonic stem cells
iPSCs	-	Induced pluripotent stem cells
TALEN	-	Transcription activator like effector nucleases
ZFN	-	Zinc finger nucleases
INS	-	Gene of insulin

INTRODUCTION

General description of the research. This Ph.D. dissertation is dedicated to studying feasibility of modulating insulin gene expression in embryonic stem cells with application of CRISPR/Cas9 genome-editing technology. Also, in vitro differentiation of these genetically modified stem cells into insulin-producing β-cells of pancreas has been performed and assessed.

Significance of the research. Diabetes mellitus (DM) is one of the most challenging medico-social problems with a heavy burden on the healthcare system. The disease is characterized by elevated blood sugar levels (hyperglycemia), which develops due to a complete lack of insulin in the body (type 1 diabetes), or due to the development of the state of "insulin resistance" or insulin deficiency (type 2 diabetes). Treatment of type 2 is targeted to normalizing the level of glycemia and preventing the state of hyperglycemia, while the situation with type 1 is more difficult to manage. The reason for the development of type 1 diabetes is the absence in the body of the most important hormone for life, insulin, caused by the complete destruction of the β-cells of the pancreas by the patient's own immune system. And the reason for the autoimmune destruction of β-cells, which normally synthesize insulin, still remains unclear.

To date, the only available treatment for type 1 diabetes is insulin injection - patients have to constantly take the hormone to maintain their life. Otherwise, blood sugar levels will keep rising, leading to a number of unfavorable consequences, including death.

The most potential treatment for type 1 diabetes is transplantation: either healthy insulin producing β-cells, the islets (Langerhans) or the entire pancreas. The last option is recommended only in exceptional cases and the criteria are the following and quite strict: very high glycemic variability (sugar level), the presence of severe autonomic dysfunction, very low quality of life due to severe complications of the disease. In any ways, in all cases the acute shortage of donor material greatly limits the opportunities. In this regard, the use of cell technology based on stem cells (SC) as a primary source is a potential solution, confirmed by the latest achievements of laboratory technics. And on top of this, capabilities of recently discovered CRISPR technology, a genome editing method, obtaining new cells with improved qualities has become relevant and could be feasible.

The purpose of the research. The purpose of the work is to create a CRISPR-based genetic approach to obtain desired modulation of insulin expression in H1 embryonic human stem cells and production of insulin-producing pancreatic β-cells derived from the CRISPR-edited H1 stem cells.

The following tasks have been identified to accomplish the purpose of the research:

1) Designing lentiviral vector containing reporter gene dsRED and two guide RNAs targeted to the promoter of the gene of insulin;

2) Obtaining two separate CRISPR-dCas9 plasmids linked with synthetic domains for activation (VP64) and repression (KRAB) of insulin transcription;

3) Validation of the effectiveness of the CRISPR-dCas9 plasmid and the

lentivector in human HEK 293 cells by identifying insulin expression. Optimization of the sequences of the constructs upon necessity;

4) Introduction of the CRISPR-dCas9-VP64 plasmid and the lentiviral vector into H1 stem cell line and testing the effectiveness of the constructs;

5) *In vitro* differentiation of the CRISPR-edited H1 cells to insulin-producing pancreatic β-cells;

6) Studying features of the obtained cells as well as identification of their glucose-responsive function.

Research objects and materials. HEK 293 cells, HEK 293T cells, H1 human embryonic stem cells, lentivirus, mouse Min6 cells, human pancreatic islets.

Research methods. Cell culture, bacterial cell culture, synthetic RNA designing, Surveyor nuclease assay, designing CRISPR-dCas9 construct, molecular cloning of fragments into constructs, constructing lentiviral vector, transduction, transfection, marker-based cell sorting, RNA isolation, cDNA synthesis, quantitative PCR, agarose gel-electrophoresis, immuno- staining, western blotting, directed differentiation of stem cells to insulin producing pancreatic β-cells, statistical analysis.

The scientific novelty of the research.

Designs of several guide RNAs (gRNAs) have been created to direct the CRISPR complex to the promoter of the insulin gene, of which 2 most effective ones were selected. A new vector has been created based on lentivirus to deliver selected gRNAs to target cells.

Plasmids with inactive nuclease dCas9 and transcription activator VP64 and transcription repressor KRAB, dCas9-VP64 and dCas9-KRAB, respectively, were obtained and validated. A stable HEK 293 cell line expressing proteins of these plasmids was obtained.

The effectiveness of the developed CRISPR complex (dCas9 nuclease, gRNA, transcription regulator) in HEK 293 cells was tested by transducing the previously obtained dCas9-VP64 HEK 293 cells with INS gRNA-containing lentiviral vector. As a result, it was shown that the developed genetic approach is able to activate the expression of insulin in these cells. Moreover, transfection of these cells with the dCas9-KRAB plasmid led to reduction in the insulin expression.

The developed constructs, in particular the CRISPR-dCas9 plasmids and the lentiviral vector, have been successfully introduced into H1 stem cells, which is confirmed by the increased expression of all key genes of each construct. Next, the combined efforts of the constructs led to increased insulin expression in the H1 cells.

In vitro directed differentiation of the genetically modified H1 stem cells into insulin-synthesizing pancreatic β-cells was performed using protocols available at the time of the research. As results of the differentiation, the final cell line expressing key markers of natural β-cells such as NKX 6.1, MAFA and insulin was obtained. Statistically significant expressions of each gene were confirmed. The resulting cells showed significantly high levels of insulin expression compared to the expression of somatostatin and glucagon.

Experimentally shown that β-cells obtained from H1 stem cells edited with

CRISPR construct have increased insulin expression compared to β-cells obtained from non-edited H1 cells. This showed that the use of CRISPR technology in the modulation of endogenous insulin in stem cells can have a positive effect on the expression of the hormone by β-cells.

Thus, the study showed the possibility of regulating insulin transcription with CRISPR-Cas9 method first in human HEK 293 cells, then in the H1 embryonic stem cells. It has also been shown that the genetically modified H1 cells can be differentiated to pancreatic β-cells, and also without losing overall gene regulation efficiency as well as expression of key genes of the CRISPR complex.

Theoretical and practical significance of the research. In the course of the research new fundamental knowledge was obtained on cell biology of HEK 293 human cells as well as on the biology of H1 stem cells. These findings hold significant theoretical knowledge in further understanding the behavior of these cells in studies using genome-editing technology.

From a practical point of view, the study revealed the possibility of developing a new potential approach of cell therapy for type I diabetes based on genome editing technology. The disease remains one of the few in the modern world that does not have a cure. Even the only available therapy to maintain patients' life - injection of exogenous insulin - leads to long-term complications. Therefore, hundreds of research groups around the world are desperately trying to find new and more effective ways to manage and treat type 1 diabetes. In this regard, the results obtained of the research and described in this dissertation will serve as new knowledge in this direction.

The main provisions for the defense.

1. Insulin gRNAs can be designed and respectively packaged into a lentivirus-based vector to be ultimately used in CRISPR gene regulation.

2. Synthetic transcription factors are feasible to be linked with deactivated Cas9 nuclease as a part of a plasmid. The linkage provides the regulator to interact with a target sequence once dCas9 interacts with the gRNA. Upon introduction in host cells, the transcription activation factor VP64 and repression domain KRAB enhances and downregulates insulin transcription, respectively;

3. CRISPR gene regulation complex consisting of the CRISPR-dCas9-VP64 plasmid and the lentivector with INS gRNA is effective in activating insulin expression in human HEK 293 cells. Addition of dCas9-KRAB plasmid to the cells causes competition between dCas9 nucleases of VP64 and KRAB for interacting with free insulin gRNAs and thus it leads to certain reduction in insulin expression level;

4. The obtained CRISPR-based approach for insulin expression regulation demonstrates similar level of effectiveness in H1 embryonic stem cells as in ordinary human HEK 293 cells. Meanwhile, those H1 cells missing insulin gRNA respectively show no level of target product expression indicating crucial role of the gRNA in CRISPR editing;

5. Directed *in vitro* differentiation of the CRISPR-edited H1 stem cells to pancreatic β-cells provides a new approach for obtaining new line of insulin-

producing cells. The obtained cells are capable of expressing more insulin than ordinary (non-genome-edited) H1-derived β-cells, but expectedly less insulin compared to natural islet cells;

6. The obtained CRISPR-edited H1-derived insulin producing cells are less sensitive to changes in glucose concentration in the media compared to natural islet cells.

Main results and conclusions:

1. Lentiviral vector containing the reporter gene dsRED and two guide RNAs targeted to the promoter of insulin has been designed and created;

2. Artificial transcription factors VP 64 and KRAB are synthetically linked with deactivated Cas9 nuclease as a part of respective plasmid. The plasmid then can be delivered into host cells for regulating target genes. Upon introduction into the host cells, the transcription activation factor VP64 and repression domain KRAB enhances and downregulates insulin transcription, respectively, when dCas9 and gRNAs interact. Stable lines of HEK 293 cells expressing dCas9-VP64 and dCas9-KRAB have been created;

3. Experimentally shown that synthetic transcription factors can activate (VP 64) and also reduce (KRAB) the expression of endogenous insulin in HEK 293 cells when used as a part of the CRISPR complex;

4. The obtained CRISPR system for insulin expression activation demonstrates enhancement in insulin production upon introduction to H1 embryonic stem cells.

5. Feasibility is shown in obtaining insulin-producing β-cells from CRISPR-modified H1 stem cells by directed *in vitro* differentiation. It is also found that the differentiation affects the efficiency of the CRISPR construct in the obtained β-cells, however adequately detectable expression of insulin remains.

6. It has been determined that the obtained CRISPR-edited H1 cells-derived insulin producing cells are less sensitive to changes in glucose concentration in the media compared to natural islet cells.

Relationship of the research with the scientific project. Majority of the research described in the dissertation was performed in the Diabetes Research Center at the University of British Columbia (Vancouver, Canada). The research was supported by the grant funding of Juvenile Diabetes Research Foundation (now – Breakthrough T1D), the project titled “Engineering tunable insulin production to mitigate stress and maximize performance”.

The research in part was performed at the laboratory of functional and structural genomics of the M.A.Aitkhozhin Institute of Molecular Biology and Biochemistry (Almaty, Kazakhstan). The research was partially supported by the funds of a grant project AP08857430 "Identification of a new minimally invasive biomarker for the diagnosis and prognostics of diabetic retinopathy based on microRNAs", supported by the Science Committee of the Ministry of Science and Higher Education of the Republic of Kazakhstan. The project was also supported by administrative resources of M.A.Aitkhozhin Institute of Molecular Biology and Biochemistry.

The contribution of the author for the results described in this dissertation. The Ph.D. candidate has performed all the studies and experiments described in this research, following the instructions (methods, protocols). All the methods mentioned above and described in this research have been developed by scientific community in the past. Some modern methods such as differentiation of stem cells to insulin producing pancreatic cells and immunostaining protocols belong to the Diabetes Research Laboratory at the University of British Columbia. The candidate by himself has performed all the analysis of the results obtained, drawing tables and figures, searching for literatures and in the writing of this dissertation.

Research approbation. The results of the research have been disseminated at the following international conferences and congresses both in Kazakhstan and abroad:

- V International Farabi Readings, 2018. Almaty, Kazakhstan;
- International Conference "International Trends in Science and Technology", 2018. Warsaw, Poland;
- International scientific conference of young scientists “Fundamental research and innovations in molecular biology, biotechnology and biochemistry” dedicated to the 80th anniversary of Academician M.A.Aitkhozhin", 2019. Almaty, Kazakhstan;
- International scientific conference of students and young scientists "Farabi Alemi", 2019. Almaty, Kazakhstan;
- VI International Congress of Young Scientists", 2019. Almaty, Kazakhstan;
- International scientific and practical conference «GLOBAL TRENDS IN THE DEVELOPMENT OF MODERN HEALTH SYSTEMS», dedicated to the 80th anniversary of the Professor Duisekeyev Amangeldy, 2022. Almaty, Kazakhstan;
- Conference “Advanced Technologies and Treatments for Diabetes”, 2023. Berlin & online, Germany;
- 1st International Forum “Asfen. Forum” held by S.D.Asfendiyarov Kazakh National Medical University, 2023. Almaty, Kazakhstan;
- The international scientific conference of young scientists «Fundamental and applied research in molecular biology, biochemistry, biotechnology», 2023. Almaty, Kazakhstan;
- Practical and patient-centered conference held by Diabetes Association of the Republic of Kazakhstan, member of International Diabetes Federation (IDF), 2023. Almaty, Kazakhstan.

Publications. The main content of the dissertation is reflected in 13 published works, including 2 articles and 1 thesis in foreign journals with an impact factor and indexed in the Web of Science and/or Scopus database, 3 articles in national scientific journals (2 of them from the list of the Committee for Quality Assurance in Science and Higher Education of the Ministry of Science and Higher Education of the Republic of Kazakhstan), as well as in the form of 1 paper and 6 abstracts in the materials of domestic and international scientific conferences.

The structure of the dissertation. The dissertation is presented on 90 pages and consists of notations and abbreviations, an introduction, a review of literature, materials and methods, results and discussion, a conclusion, a list of used literature sources of 169 titles, and also contains 32 figures.

1 LITERATURE REVIEW

1.1. Diabetes Mellitus Type I

Diabetes mellitus (DM) type 1 is a polygenic and multifactorial disease, which develops due to immune-mediated or idiopathic destruction of pancreatic β-cells leading to absolute deficiency of insulin [1].

Genetic predisposition is a key factor in the development of diabetes type 1, but at the same time it is environmental factors that act as a triggering agents that together will lead to autoimmune damage of pancreatic β-cells [2]. An environmental factor can be both infectious and non-infectious [3,4]. Typically, viruses (retroviruses and enteroviruses,) act as infectious triggers while non-infectious can be diverse: glucose, heavy metals, dietary components (gluten, soy), cow's milk or breast milk, psychosocial factors (stress), unsaturated fats, ultraviolet radiation, antioxidants, seasonal change in temperature or any toxic agent for β-cells.

Usually, detection of islet antibodies causes changes in insulin secretion as well as glucose tolerance and this period takes from one to three months. In most cases, when the disease is diagnosed a large mass of β-cells is already destroyed (up to 90%), leading to immediate need of exogenous insulin administration. When the symptoms occur to be visible it means that "latent phase" is passed which lasts from several months to many years.

Triggering factors of the disease might either (1) activate polyclonal lymphocytes (for example, by infectious agents) or (2) increase immunogenicity inducing an immune response or even work by (3) mimicking – displaying identical sections of the protein sequences of an infectious or chemical agent to autoantigens. Any of these ways will ultimately be enough to trigger the development of autoimmune processes and unfavorably lead to the production of various autoantibodies such as autoantibodies to insulin (IAA), islet cells (ICA), zinc transporter (Zn-T8A), glutamate decarboxylase (GADA) and tyrosine phosphatase-like protein (IA-2A) [5,6].

1.1.1. Clinical image and pathogenesis of the disease

Typically, diabetes type 1 has an acute onset followed by rapid development of different metabolic disorders. The disease usually shows up in early years of life such as childhood and adolescence, however there are also cases when it can possibly start to develop at any age, including even old age. According to findings to date, type 1 diabetes is often associated with other autoimmune diseases [7].

Genetic predisposition is one of the key factors and sometimes even necessary for the development of the disease, but not all the disease cases have it. Based on study results, in families of type 1 diabetic patients' brothers, sisters, children are parents are found to be at the highest risk of developing it in the future.

There is the preclinical stage of the disease when antibodies can be detected even before the appearance of the first clinical symptoms. Viral infection, stress, or overload of the body with easily digestible carbohydrates can act as those triggers that accelerate the clinical stage development of type 1 diabetes.

The clinical presentation of the disease is caused by absolute insulin deficiency and might vary from moderate or severe signs of hyperglycemia to severe dehydration, diabetic ketoacidosis (DKA) or even up to the development of a coma.

Usually, symptoms of classic type 1 diabetes develop quite quickly. For instance, after 2-4 weeks of an infection patients start to experience dry mouth, thirst up to 3-5 L/day (polydipsia), increased appetite (polyphagia), polyuria at night. If to do screening, hyperglycemia and glucosuria will be found. Hyperglycemia, which in turn leads to glucosuria, is caused by a decrease in glucose utilization by peripheral tissues due to a lack of insulin.

It is known that glucose is completely reabsorbed in the tubules of the kidneys when its concentration in blood plasma is up to 10 mmol/l and exceeding this threshold leads to the excretion of glucose into the urine. Polyuria, which is typically accepted as the first symptom of glucosuria, usually accompanies high glucosuria and it is a consequence of osmotic diuresis. Dry mouth and thirst are associated with dehydration due to excessive excretion of fluid through the kidneys and also due to an increase in levels of glucose, urea, and sodium in the blood. During polyuria urine gets colorless and might be sever up to 3 liters per day. Polydipsia is a result of irritation of the thirst center in the brain due to hyperosmolarity of the blood as well as dehydration. The feeling of thirsty is more specific to night and early morning times.

Severe weight loss and weakness usually follow the clinical symptoms of type 1 diabetes. Acute insulin deficiency leads to total carbohydrate metabolism disorders which in turn cause imbalances of protein and fat metabolism. This changes lead to hyperaminoacidemia, hyperlipidemia, and ketoacidosis. Insulin deficiency stimulates gluconeogenesis and glycogenolysis, but at the same time suppresses glycogenogenesis in the liver.

Increases in the levels of cholesterol, triglycerides and lipoproteins is the clear sign of hyperlipidemia. The hyperlipidemia causes increased intake of lipids in the liver, where they get intensely oxidized leading to an increase in the production of ketone bodies (acetone, β-hydroxybutyric and acetoacetic acids) and hyperketonemia. This causes diabetic ketoacidosis, a decrease in pH and the development of tissue hypoxia. The development of all the mentioned metabolic disorders caused by insulin deficiency leads to tissue dehydration, hypovolemia, hemoconcentration with a tendency to develop disseminated intravascular coagulation, hypoxia, and cerebral cortex edema and, ultimately, the development of diabetic coma.

It is not necessary that all diabetes type 1 patients immediately start feeling the increased level of glucose in the blood plasma, some patients might not have any symptom. However, as the clinical practice showcases, those patients who have a symptom usually complain about weakness, headache, and sometimes visual impairment. The reason for the decrease in visual function is the swelling of the lens caused by hyperglycemia. Polyuria and thirst, caused by a high content of glucose in the blood plasma, can be observed not in every patient with metabolic decompensation [8].

In terms of the pathogenesis, the theory brought in by Eisenbarth has been the most widely accepted one over the past 35 years [9]. Genetically predisposed people were thought to be as those who would develop the disease, based on the theory, and external factors have been meant to be causing autoimmunity processes. Death of islet cells, which is the starting phase of the type 1 diabetes, progresses without symptoms. The clinical symptoms appear at final stages of islet cells destruction where majority of beta cells have already died, and insulin deficiency taken place.

In the beginning, the genetics of type I diabetes seemed to be simple – human leukocyte antigen (HLA) system's genes were thought to be main driver of the disease [10]. The studies earlier done by Nerup and others in 1970s clearly showed the connection between type 1 and HLA system [11]. To date, it is known that there are over 20 loci, and 100 genes-candidates associated with pathogenesis of type 1 diabetes [12,13].

1.1.2. Diagnostics of type I diabetes

Like other types of the disease, diagnostics of type 1 is carried out in a clinical laboratory by determining the level of glycemia (blood glucose level). The diagnosis of the disease should always be confirmed by repeated glycemic testing on subsequent days, except in cases of unequivocal hyperglycemia with acute metabolic decompensation or obvious symptoms. The diagnosis of gestational diabetes can be made on the basis of a single determination of glycemia. According to World Health Organization (WHO), in normal health conditions on an empty stomach the level blood glucose is always less than 5,6 mmol/L in whole capillary blood, while in venous plasma it is always below 6,1 mmol/L. It is considered to diabetic state if whole capillary blood's glucose level is no less than 6,1 mmol/L or venous plasma's glucose level is no less than 7 mmol/L [14,15]. Conversion of blood glucose from mmol/L to mg/dL can be calculated as mmol/L × 18.02 = mg/dL.

1.1.3. Treatment. Insulin therapy.

Nowadays, the treatment of diabetes type 1 consists of the following: insulin replacement therapy, self-control of glycemia and teaching patients and their families for principles of disease management.

Insulin replacement therapy is the only treatment for patients with type 1 diabetes. For all patients with type 1 diabetes, the first-line drugs are ultrashort-acting, long-acting, and extra-long-acting human insulin analogues. It is recommended to prescribe intensified insulin therapy by multiple injections and basal insulin or by continuous subcutaneous insulin infusion in patients with type 1 diabetes to achieve the targets of glycemic control [16].

It is also highly recommended to determine the correspondence of the dose of insulin to carbohydrate intake, blood glucose levels before meals and expected physical activity in patients with type 1 diabetes to improve glycemic control.

The use of insulin pumps in patients with type 1 diabetes is recommended when individual targets for glycemic control are not achieved against the background of intensified insulin therapy in a multiple injection regimen and/or a

decrease in quality of life in order to optimize treatment [17-19].

There are currently no surgical treatments for type 1 diabetes. Isolated pancreas transplantation in patients with type 1 diabetes is not recommended for the treatment of the disease [20]. Criteria for inclusion of patients in the list for pancreas-only transplantation were recognized in the following cases: very high glycemic variability, the presence of severe autonomic dysfunction, or a very low quality of life due to severe disease complications [21]. Isolated pancreas transplantation is currently not performed in patients with type 1 diabetes before the development of end-stage renal disease, as the surgical risk, the likelihood of rejection, and the consequences of immunosuppressive therapy are so great that they significantly reduce the life expectancy and quality of life of patients [22].

For self-monitoring of blood glucose levels, it is recommended to use glucometers designed for individual use, including those with setting an individual target range of glycemia, as well as transmitting glucose level data to a smartphone, from where they can be used for remote monitoring. When plasma glucose is <5.6 mmol/L, 95% of measurements must deviate from the reference analyzer by no more than ± 0.8 mmol/L, when plasma glucose is ≥5.6 mmol/L, 95% of measurements must deviate from reference analyzer by no more than ±15%.

Education is considered as an integral part of the complex of therapeutic measures for type 1 diabetes and should continue throughout the course of the disease. Treatment of DM 1 necessarily includes training in self-monitoring of glycemia, the principles of disease management (including adaptation of insulin doses).

Most of the time in the teaching process should be devoted to the practical development of the skills necessary for self-management of the disease. First of all, this should include general information about diabetes, nutrition, physical activity, self-monitoring of glycemia (blood glucose levels), insulin therapy, hypoglycemia, late complications of diabetes, follow-up examinations in diabetes, insulin injection techniques, rules for adjusting insulin doses, foot care, self-measurement of blood pressure.

1.1.4. Potential future treatment options

Insulin replacement therapy is a way of supporting life of a patient with type I diabetes and to date it is the only possible way of keeping the patient alive and managing the disease. There is no cure for the disease. Nevertheless, now there are many studies are under completion in search of better understanding the pathogenesis of the disease and at the same time trying to come up with treatment. As it was described earlier, during diabetes type 1 the beta cells of the endocrine part of the pancreas is lost. Since there is a loss and considering the current understanding of the disease, transplantation is the only doable, promising, and worth treatment of this pathology. Replacing lost β-cells with functional and healthy cells could be the future of the treatment. However, the lack of donor materials worldwide for clinical cell transplantation greatly limits this therapy [23,24], thus production of β-cells (or β-like cells) from other cell types is now considered as the most promising approach.

As one potential solution within the approach is the use of embryonic stem cells (ESCs). ESCs could be of unlimited supply of β-cells, but there is still no proper differentiation protocol although numerous research groups have been working on it for decades [25-28].

1.2. Genome editing technologies

Genome editing (also known as "gene editing") is a way of modifying the DNA of living organisms such as bacteria, viruses, plants, animals, and humans. Nowadays, genome editing methods and approaches are widely used in many scientific studies aimed at studying fundamental biological processes as well as seeking treatment options.

Genome editing marks one of the new areas of genetic engineering that is truly revolutionary in biological medicine. Over the past decades, various genome editing technologies have been developed: zinc finger nucleases (ZFN), transcription activator like effector nucleases (TALEN), and clustered regularly spaced short palindromic repeats recognized by the Cas9 nuclease (CRISPR/Cas9). The most widely known and used method is the CRISPR/Cas9 system, which has many advantages over other existing genome editing systems.

The first generation of genome editing tools were ZFNs, protein complexes containing a certain type of protein domains - "zinc fingers", which contain a zinc molecule and structurally shaped like a finger. Each of these protein domains can recognize and specifically binding to a specific three-nucleotide sequence of a DNA molecule. By creating artificial nucleases, for point impact on the areas of interest in the genome, a chain of “zinc fingers” is constructed in a way so that it specifically recognizes and binds to the exact DNA site that is called “target sequence”. This method has not been widely used, since the recognition of repeats consisting of three nucleotides is not strict, which can lead, in some cases, to cutting the DNA molecule in “non-target” areas. In addition, the method is relatively time-consuming and expensive because for each DNA sequence of interest it is necessary to create a specific protein structure of the nuclease.

Comparing to ZFNs, constructs based on chimeric nucleases called TALENs was found to be a more promising means of selective action on DNA. They were based on transcription activator-like effector proteins (TALE), which identify and trigger promoters of genes using a set of tandem repeats. TALEN, which is an artificial nuclease, consists of two functional domains: a DNA recognition domain and a non-specific *Fok I* DNA cleavage domain. The role of the DNA-recognizing structure in this system is played by protein domains (TALE), which, in turn, include the central repeating domain (CRD). It provides DNA binding and consists of tandem repeats (contains 34 amino acid residues), each of which "recognizes" only one nucleotide in the target nucleotide sequence. The two amino acid residues in the repeat located at positions 12 and 13 are highly variable (repeated variable direction - RVD) and are responsible for specific nucleotide recognition with multi-nucleotide binding degeneracy with differential efficiency. Another component of chimeric nucleases is the catalytic domain of the Fok I restriction endonuclease. Later, it

became possible to create and use artificial nucleases with a DNA-binding domain and various RVDs that can target any nucleotide sequence of interest to the researcher.

The use of mega nucleases such as ZFNs and TALENs is based on the complex design and synthesis of proteins with tunable DNA-binding specificity which limits their distribution and use.

One of genome editing methods that appeared not so long ago is the use of Clustered Regularly Interspaced Short Palindromic Repeats - CRISPR, where best known system is CRISPR/Cas9. This method uses the adaptive immune system of bacteria and archaea, the mechanism of which depends on the presence of special regions in the genome, called CRISPR loci [29].

1.2.1. Current state of research on genome editing technologies used in diabetes studies

CRISPR/Cas technology has emerged as a promising tool in the treatment of Type 1 Diabetes (T1D) by enabling precise genome editing. This technology is being explored for its potential to engineer insulin-producing β-cells and modulate immune responses to prevent the autoimmune destruction characteristic of T1D. However, challenges such as the risk of off-target effects and the complexity of safely engineering cells remain significant hurdles [30-32]. CRISPR/Cas9 has also been used to guide mesenchymal stem cells (MSCs) towards differentiation into insulin-producing cells, offering a novel therapeutic avenue.

In addition to CRISPR, other genome editing technologies such as Zinc Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs) are being explored for their potential in diabetes treatment. These technologies offer alternative methods for precise genome modification, although CRISPR/Cas9 remains the most widely studied due to its simplicity and efficiency [33].

Gene therapy is being investigated as a potential treatment for T1D, focusing on the overexpression of protective genes and the transplantation of genetically modified cells. These strategies aim to restore normoglycemia and induce immune tolerance to prevent further β-cell destruction. However, the safety and efficacy of these therapies in humans are still under investigation, with most studies currently in preclinical stages. Cell-based therapies, including the use of bioengineered islets from stem cells, are also being explored to replace damaged β-cells and restore insulin production [34].

The results of genetic engineering research, from the production of recombinant human insulin to the implantation of insulin-producing cells derived from the patient's own cells, suggests that the development of number of effective treatments for insulin-dependent diabetes is becoming a reality.

In summary, genome engineering technologies, particularly CRISPR/Cas9, are at the forefront of innovative diabetes treatments. These approaches offer the potential to transform diabetes management by enabling precise genetic modifications and developing novel cell-based therapies. However, further research is essential to overcome current limitations and translate these promising

technologies into effective clinical treatments.

1.3. CRISPR system

The CRISPR system is based on special sections of bacterial DNA which are short palindromic cluster repeats or *CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)*. Between identical repeats there are DNA fragments differing from each other - spacers, many of which correspond to sections of the genomes of viruses that parasitize on this bacterium. When a virus enters a bacterial cell, it is detected using specialized Cas proteins (CRISPR-associated sequence) associated with CRISPR RNA. If a virus fragment is "recorded" in the CRISPR RNA spacer, Cas proteins cut the target viral DNA and destroy it, protecting the cell from infection [35].

No one could have imagined that the practical possibility of treating human genetic diseases would appear due to bacteria. In the late 80s, Japanese scientists partially sequenced the E. coli genome and found a region that did not encode anything. This region contained repetitive DNA sequences separated by variable regions called spacers. The presence of a long non-coding region surprised the group since bacteria are frugal with their DNA and usually do not carry extra sequences. Later, similar "cassettes" of repeats and spacers were found in a large number of bacteria and archaea and were called CRISPR.

In the early 2000s, several scientists independently compared the sequences of known CRISPR spacers with DNA sequences deposited in public databases. It turned out that quite often the sequences of spacers were similar to those of viruses. This suggested that CRISPR cassettes may have a protective function. At the same time, Cas genes were discovered, often located next to CRISPR cassettes. The group of scientists proposed a rather detailed hypothetical scheme of the mechanism of action of CRISPR/Cas systems. According to their model, when a virus enters a cell, it is detected using the Cas protein, which uses an RNA copy synthesized with CRISPR. If any fragment of the virus genome matches that recorded in the spacer, Cas cuts the viral DNA and starts a chain of reactions, as a result, all DNA is destroyed.

Later it was found that in the CRISPR cassettes of clones that became resistant to the virus, new spacers appeared that corresponded to regions of the viral genome. The scientists, using the methods of molecular genetics, inserted a spacer with the DNA sequence of the virus into the CRISPR cassette of the bacterium. And such a genetically modified bacterium turned out to be resistant to the virus [36].

1.3.1. Development of CRISPR/Cas9 technology

In 2012, the group of E. Charpentier and J. Doudna published a joint paper in *Science*, where they proposed a way to reprogram the CRISPR/Cas system so that it began to cut DNA in a targeted way at the sites chosen by the researcher. In nature, CRISPR RNA is encoded in a CRISPR cassette, bound by proteins, and then recognizes the target. It was found that it is possible to obtain non-natural CRISPR RNA using chemical or enzymatic synthesis. In this case, the place of the spacer in

such RNA is occupied by the sequence chosen by the researcher. The Cas9 protein is able to “recognize” and bind to such a synthetic CRISPR RNA (it is called a “guide”) and becomes programmed to recognize and cut its corresponding place in DNA. Charpentier and Doudna's group demonstrated the feasibility of this approach in vitro.

CRISPR/Cas9 technology consists of a gRNA molecule for targeting and Cas9 nuclease for cleavage. Cas9 comes from *Streptococcus pyogenes* and functions to protect the bacteria from foreign bodies by destroying their DNA [37]. In nature, these bacteria have two separate RNA molecules (crRNA - transactivating CRISPR RNA and pre-crRNA - precursor CRISPR RNA) that by acting together direct Cas9 to a target (for example, phage). However, in research scientists usually synthetically join the two as one chimeric guide RNA (gRNA). The gRNA forms a ribonucleoprotein complex with the Cas9. Several research labs have previously shown that expression of type *II* CRISPR/Cas9 system in mammalian cells leads to the formation of double-strand brakes (DSBs) at target sites: the gRNA’s protospacer region matches with a 20-bp sequence of the target DNA and as a result, Cas9 cleaves the DNA [38].

In last years, CRISPR/Cas9 system has gained superior attention: the target specificity of Cas9 derives from RNA:DNA complementarity while TALENs and ZFNs require modifications to the protein itself to target DNA [39]. CRISPR/Cas9 can be easily adapted to target any genomic sequence just by changing the protospacer of gRNA. The Cas9 protein component remains unchanged. This easy programmability of CRISPR/Cas9 system is a significant advantage over ZFNs and TALENs, which require tremendous amount of bench work.

1.3.2. Guide RNAs in CRISPR/Cas9 systems

As mentioned above, guide RNAs (gRNAs) act as an agent driving the CRISPR complex to the target sequence. In nature, Cas 9 nuclease needs two RNAs which by acting together directs the enzyme: crispr RNA (crRNA) and trans RNA (trRNA). In experiments, for the ease of the studies researchers have synthetically joined the two RNAs and have made one guide RNA. Thus, gRNAs are essential components of CRISPR gene regulation systems, directing the CRISPR protein effectors to specific genomic targets. Recent advancements in the engineering of gRNAs have expanded their functionality, enabling precise control over gene expression and editing [40].

Engineered gRNAs can improve the specificity and efficiency of CRISPR systems. Modifications such as chemical alterations, spacer length adjustments, and fusion with RNA or DNA components enhance target specificity and reduce off-target effects, which is crucial for safe and effective gene therapy [41].

Multiplexed CRISPR technologies allow for the simultaneous expression of multiple gRNAs, enabling complex genetic circuits and large-scale genome engineering. This approach is beneficial for applications such as cellular recorders, biosensors, and for metabolic pathway rewiring. Also, some engineered gRNAs enable dual-purpose CRISPR systems that can perform both gene editing and

regulation. For example, truncating gRNA spacer length can inhibit nuclease activity while allowing binding, facilitating gene activation and repression [42,43].

Efficient expression of gRNAs remains a challenge, particularly in new host organisms. Strategies such as using T7 polymerase for gRNA expression have been developed to overcome these limitations, allowing for high-efficiency CRISPR editing in various yeast hosts [44].

To date, engineering of gRNAs has significantly advanced the capabilities of CRISPR systems, enabling precise, efficient, and versatile gene regulation and editing. These innovations hold promise for a wide range of applications in synthetic biology, therapeutic development, and beyond.

1.3.3. CRISPR/Cas9 technology in diabetes research

To date, CRISPR-Cas9 technology has been used in diabetes research in a variety of ways, but mainly for precise editing of individual genes. Such examples were the perturbation and study of type 2 diabetes risk genes [45], creating of engineered brown adipocyte cells for improving glucose tolerance and insulin sensitivity [46], and CRISPR-based genome editing of stem cells to fix related mutations and maintain immunosuppression after transplantation of insulin-producing cells [47].

There are examples where CRISPR editing have been as a tool to learn diabetes-related science questions. Particularly, Fang and his team found out that the cohesin complex and the NuA4/Tip60 histone acetyltransferase complex modulate insulin gene expression using intracellular insulin immunofluorescence as a quantitative readout [48]. Wei et al. produced β-like cells from iPS cells through differentiation and explained that vitamin D receptor (VDR) acts as a regulator of inflammation and β-cell survival [49].

Genome-wide CRISPR knockout screen was used to identify genes that regulate pancreatic β-cell protection against autoimmune destruction during type 1 diabetes development. It was found that autoimmune destruction causes a strong live-death selection pressure on transplanted pancreatic β-cells. Moreover, it was discovered that some gene mutations and a human type 1 diabetes GWAS gene reduce intrinsic stress on β-cells' function and protect the transplanted cells from the autoimmunity [50].

1.4. Synthetic transcription factors

Synthetic transcription factors (STFs) are artificial or naturally existing molecules whose primary function is not related to transcription process in nature. These transcription factors are composed of a DNA-binding domain (DBD) that recognizes specific DNA sequences and one or more transcriptional effector domains (TEDs) that exert transcriptional changes. While natural transcription factors often refer to transcription activators, historically the term STF has been used to describe both activators and repressors [51]. The development of STFs provides a powerful toolkit for gene and cell therapy via effective, precise, and controllable modulation of endogenous or exogenous therapeutic genes. These molecules can

alter transcription of genes and other sequences as a part of CRISPR complex.

CRISPR-based synthetic transcription factors utilize guide RNAs (gRNAs) to direct the CRISPR/Cas system to specific DNA sequences, allowing for targeted gene activation or repression. This method simplifies the process of transcriptional regulation compared to traditional DNA-binding protein engineering, which requires complex construction and optimization [52,53]. The CRISPR/Cas9 system, in particular, has been adapted to create RNA-guided transactivators that can induce specific gene expression in human cells.

The use of CRISPR-based synthetic TFs has been demonstrated in various applications, including the modulation of endogenous gene expression in vivo, which holds potential for therapeutic cellular programming [54]. These tools have been applied to upregulate gene expression in mammalian cells, with systems like CRISPR/Cas12a offering multiplexed activation capabilities [55].

1.4.1. Synthetic transcription activators

Prior to the discovery of CRISPR/Cas systems, gene activation across multiple loci was an arduous process. When using zinc finger proteins or TALE proteins, proteins had to be re-engineered for each gene, making wide-scale gene activation seem next to impossible. The development of CRISPR/Cas systems, however, greatly improved the simplicity of gene activation: rather than requiring protein engineering for each locus, CRISPR/Cas systems only require changing the programmable guide RNA.

Among other synthetic molecules, VP64 is nowadays the most widely used one with the CRISPR complex. Guided by dCas9 nuclease of the complex, VP64 recruits transcriptional machinery to specific sequences, causing targeted gene regulation. This can be used to activate transcription during either initiation or elongation, depending on which sequence is targeted [56].

Synergistic Activation Mediator (SAM) uses specially engineered sgRNAs to increase transcription. This is done through creating a dCas9-VP64 fusion protein engineered with aptamers that bind to MS2 proteins. These MS2 proteins then recruit additional activation domains such as HS1 and p65. When targeting single genes, SAM consistently relatively high levels of gene activation compared to other CRISPR activators, making it as popular as the VP64 for gene activation experiments. In cases of multiplex gene regulation (activating multiple genes at once), SAM exhibits activation levels comparable to other popular activation methods such as VPR and SunTag [57].

Transcription activator SunTag's mechanism of action is similar to VP64. The main difference is that SunTag uses a repeating peptide array fused with multiple copies of VP64. Consequently, having multiple copies of VP64 at each locus of interest, it allows more transcriptional machinery to be recruited per targeted gene [58]. However, one disadvantage of this method is that it relies on antibody chains, which are relatively large and are not expressed consistently throughout cells.

VPR factor fuses a tripartite complex with dCas9 to activate transcription of the target sequences. This complex consists of the VP64 activator used in other

CRISPR activation methods as well as two other potent transcriptional activators p65 and Rta. These transcriptional activators work in tandem to recruit transcription factors [59]. An advantage to this method compared to other notable CRISPR activators is that it requires a fusion protein rather than relying on a two-component system dependent on gRNA design (SAM) or peptide design (SunTag) [60]. This streamlines its delivery, making it a common choice for CRISPR activation.

1.4.2. Transcription repressors

To date, there are a few well known synthetic transcription repressors though in comparison to activators they are relatively less used. The most widely known one is Krüppel associated box (KRAB). The KRAB is a category of transcriptional repression domains present in approximately 400 human zinc finger protein-based transcription factors. The KRAB domain typically consists of about 75 amino acid residues, while the minimal repression module is approximately 45 amino acid residues. It functions through protein-protein interactions via two amphipathic helices. The most prominent interacting protein is called TRIM28 initially visualized as SMP1, cloned as KAP1 and TIF1-beta. Substitutions for the conserved residues leads to losing repression effect. Over 10 independently encoded KRAB domains have been shown to be effective repressors of transcription, suggesting this activity to be a common property of the domain. KRAB domains can be fused with dCas9/CRISPR tools to form even stronger repressors [61,62].

Histone deacetylase inhibitors (HDIs) are relatively any molecules that inhibit the respective enzymes - histone deacetylases. Since deacetylation of histones produces transcriptionally silenced heterochromatin, HDIs can render chromatin more transcriptionally active and induce relevant changes. Over the latest decade, in general, HDIs have been studied as promising agents for effective modulation of transcriptional sequences in science [63-65].

Anyway, synthetic transcription factors integrated with CRISPR technology offer a powerful tool for precise gene regulation, with significant implications for research and therapeutic applications. While challenges remain, ongoing advancements in this field promise to enhance our ability to manipulate gene expression and develop novel treatments for various diseases.

1.5. Cloning vectors in CRISPR gene regulation systems

Molecular cloning vectors are essential tools in CRISPR gene regulation, enabling precise genetic modifications and functional studies. These vectors facilitate the delivery and expression of CRISPR components, such as Cas9 and guide RNAs (gRNAs), to target specific genomic loci for gene editing or regulation.

In the context of gene regulation using CRISPR, several molecular cloning vectors are widely used to facilitate the delivery and expression of CRISPR components. These vectors are designed to optimize the efficiency and specificity of CRISPR-mediated gene editing [66, 67].

The most widely used molecular cloning vectors for CRISPR-based gene regulation include adenovirus, AAV, and lentiviral vectors, each offering unique

advantages for specific applications. Inducible and plant-specific vectors, along with multiplexing systems, further expand the versatility and efficiency of CRISPR technology in various research and therapeutic contexts. These tools collectively enhance the precision and scalability of CRISPR-mediated gene regulation.

Adenovirus vectors, such as the AdZ-CRISPR, are optimized for high-throughput cloning of sgRNA and Cas9 sequences. These vectors are particularly effective for gene knockout, achieving up to 80% efficiency with a single sgRNA, and are suitable for hard-to-transfect cells [68].

AAV (Adeno-Associated Viral) vectors have been utilized to deliver CRISPR-Cas9 systems for in vivo gene regulation. These vectors enable targeted gene repression and activation without permanent genome modification, as demonstrated in studies targeting diseases like retinitis pigmentosa. The modular nature of AAV vectors allows for robust transcriptional control, making them suitable for therapeutic applications [69,70].

Plasmid vectors, such as those developed using the ASAP-cloning and STAgR systems, enable the assembly of multiple gRNA expression cassettes in a single step. These systems are adaptable, allowing for the customization of vector backbones and gRNA structures, and are suitable for multiplex CRISPR applications [71].

Golden Gate cloning is a popular method for constructing vectors with multiple gRNAs, particularly in plant genome editing. This method uses restriction enzymes to assemble gRNA polymers, allowing for the targeting of multigene families or multiple genes simultaneously [72]. In addition to the Golden Gate, a number of one-step cloning systems have been developed to simplify the generation of CRISPR vectors. These systems, such as the inducible CRISPR/Cas9 platform and the Fragmid toolkit, allow for rapid and cost-effective assembly of CRISPR components, facilitating multiplex gene editing and reducing off-target effects.

The STAgR system provides a single-step method for generating customizable gRNA vectors, enabling efficient multiplex CRISPR approaches. This system is adaptable, allowing for the combination of different vector backbones, gRNA structures, and promoters, which is crucial for complex gene regulation tasks [73,74].

A key challenge in CRISPR vector design is achieving efficient multiplexing while minimizing off-target effects. Advances in vector design, such as the use of inducible systems and optimized sgRNA sequences, are addressing these challenges by improving specificity and control over gene editing processes [75].

The development of modular and customizable vector systems is crucial for expanding the applications of CRISPR technology. These systems allow researchers to rapidly test and optimize new CRISPR technologies, enhancing the versatility and applicability of CRISPR in various biological contexts [76].

Ultimately, the development of diverse and efficient molecular cloning vectors is enhancing the capabilities of CRISPR/Cas9 technology, enabling precise and versatile genome editing across different organisms and applications.

1.6. Stem cells

Stem cells (SC) are an individual cell or a group of progenitor cells with the ability to self-renew and differentiate into specialized tissues. Therefore, from the beginning of its discovery in early 1900s, SCs have become one of the most promising research areas in biomedical science. Currently, stem cells are divided into three categories depending on their origin: embryonic, fetal, and adult stem cells [77].

Embryonic stem cells (ESCs) are ones isolated from early embryos (at the blastocyst stage or from the germinal germ of 5-week-old embryos) or teratocarcinoma (tumor line) in vitro.

ESC, in turn, is divided into the following types:

1. Totipotent - cells of embryos and extra-embryonic cell membranes before implantation (11 days after fertilization), capable of differentiating into a full-fledged organism.

2. Polypotent - cells of the embryo from the post-implantation period up to 8 weeks inclusive, capable of differentiating into a complete organ or tissue structure.

Currently, research is being actively conducted in the field of obtaining pluripotent somatic cells of animals and humans. In principle, two ways are described by which this can be achieved. This is the use of cloning methods (for example, cell fusion, transfer of somatic cell nuclei into an oocyte of the second division of meiosis (somatic cell nuclear transfer, SCNT) [78] and induction of reprogramming with the production of induced pluripotent stem cells (iPS cells), similar to with ESCs [79]. Japanese scientists for the first time succeeded in obtaining SCs that do not differ in their properties from embryonic SCs and that can transform into cells of any tissues of the mammalian organism. Connective tissue cells (fibroblasts), taken from mouse embryos, as well as cells of adult animals were used in the experiment to generate iPSCs [80].

In adult organism, there are some types of stem cells can be found:

1. Hematopoietic stem cells located in the hematopoietic organs and blood, capable of giving rise, mainly, to various hematopoietic sprouts.

2. Mesenchymal (stromal) stem cells located in the bone marrow, with the ability to differentiate into osteoblasts, chondrocytes, tenocytes, adipocytes, myoblasts, fibroblasts.

3. Stem cells of other tissues (regional), for example, skin, blood vessels, nervous tissue, and others, are located in the corresponding tissues and differentiate into cells of these tissues.

1.6.1. Stem cells and diabetes. Differentiation of stem cells into pancreatic β-cells

Process of transformation of stem cells into desired cell type is called differentiation. Currently, the stem cell differentiation technique provides many opportunities for cell therapy of pathologies, such as diabetes, caused by a violation of cells of one type. Many types of stem cells have been studied, but embryonic stem cells (ESCs) are considered the most promising, since they have an almost unlimited

proliferative capacity and can differentiate into almost any type of somatic cells [81,82].

ESCs are an alternative source of highly proliferative pluripotent cells that have received much attention. Derived from a blastocyst-stage embryo, these cells in vitro have the ability to differentiate into all three germ layers. The first attempts to differentiate ESCs into β-cells were due to the advantage in the selection and subsequent growth of undifferentiated cells that spontaneously expressed insulin, nestin [83], but the amount of insulin obtained in this case was very small. A significant step forward was made by the Baetge group, which, in order to develop a differentiation protocol, investigated signals that stimulate development and are capable of inducing pancreatic organogenesis in vivo, which ultimately should allow the first identified endodermal cells to be obtained from human ESCs [84] and then cells that produce insulin [85]. Using this five-stage differentiation protocol, which corresponds to each of the stages of pancreatic formation, the authors were able to achieve the formation of approximately 7% of cells capable of producing insulin in vitro.

Blyszczuk et al. described an efficient technique for in vitro differentiation of ESCs into a pancreatic lineage. Differentiated cells secrete insulin in response to high glucose levels. However, in another study it was shown that such cells secrete insulin under the action of various agents that enhance its secretion, but do not respond to glucose stimulation [86].

Later Baetge and colleagues improved their results by optimizing the in vitro differentiation protocol and by transplanting ESC-derived pancreatic progenitors into mice so that after three months in vivo, the implanted cells differentiated into mature endocrine cells capable of regulating glucose levels [87]. The same group of researchers, by further optimizing the differentiation protocol for the CyT49 ESC line, developed a scalable and standardized system for obtaining functional progenitor cells from human ESCs, which was also a big step towards clinical implementation [88]. Despite significant advances, three major problems limit the applicability of insulin-producing cells derived from ESCs. First of all, due to the fact that these cells are pluripotent, undifferentiated cells in vivo serve as a source for the development of teratomas, and their transplantation will inevitably lead to the formation of a tumor due to the presence of some residual number of undifferentiated cells. Several attempts have been made to search for surface markers that would allow the selection of pancreatic progenitor cells [89] or the removal of only pluripotent cells [90], but the safety of selected cells also needs to be further studied.

It is obvious that many of the principles revealed in mouse ESC cells cannot be directly applied to human ones. Moreover, under physiological conditions, β-cells are located in a complex islet structure, which allows control of insulin incretion through various mechanisms: neural (for example, sympathetic nerve fibers), endocrine (for example, glucagon) and paracrine (for example, somatostatin). In addition, ethical issues associated with obtaining insulin-producing cells from human embryos complicate the further development of this direction.

In 2006, a possible solution to issues associated with the use of ESCs appeared when Yamanaka and colleagues managed to reprogram the development of somatic cells in adult mice [91] and later in adult human [92] by means of forced expression of 4 genes (OCT4, SOX2, KLF4 and c-MYC) with the formation of induced pluripotent stem cells (iPSC). These cells retain the basic properties of ESCs, such as pluripotency and the ability to self-sustain, but at the same time provide the opportunity for the formation of autologous cells, which can be used for cell therapy. Recently, human iPSCs have been generated by reprogramming many types of somatic cells, with many studies reporting successful differentiation of these cells into neurons, cardiomyocytes, hepatocytes, or hematopoietic cells [93]; however, differentiated cells derived from iPSCs may also be useful in in vitro disease modeling and/or drug research. Thus, these cells can serve as an alternative and more powerful source of stem cells used to treat various diseases, including type 1 diabetes. In 2008, thee first report came out on successful differentiation of iPSCs into insulin-producing cells using the four-step protocol described for ESC differentiation [94].

Impressive results have been reported in several in vitro studies in which the authors used other protocols mimicking the mechanisms of pancreatic development in vivo to direct iPSC differentiation into β-like cells [95-98]. Insulin-producing cells were also obtained from iPSCs formed by reprogramming fibroblasts from two diabetic patients [99].

Human iPSCs have also been obtained by reprogramming pancreatic β-cells and then re-differentiating into insulin-producing cells that are more efficient than differentiation of iPSCs derived from reprogramming non-β-cells of that same patient [100]. The results of this work show that iPSCs have the epigenetic memory of the original cell even after reprogramming, and that not only ESCs, but also iPSC cell lines are characterized by varying degrees of ability to differentiate into β-cells.

The main concern with using iPSCs is their safety. In fact, in addition to oncogenic properties due to pluripotency, the use of oncogenes for reprogramming, as well as the fact that oncogenes are irreversibly integrated into the cell genome (due to the use of retroviruses and lentiviruses), can cause the formation of malignant neoplasms.

In general, it should be said that great hopes are pinned on iPSC cells in the framework of cell replacement therapy for DM, but a lot of research is still needed to increase the safety and efficiency of reprogramming and differentiation processes.

To date, there are a number of growth factors and agents used in differentiation of iPSCs to insulin producing cells in vitro [101-106]. Common to all methods is the use of activin A and retinoic acid. The action of activin A on differentiated cells leads to differentiation of the endoderm, subsequent treatment with retinoic acid leads to the induction of differentiation along the pancreatic pathway. Both inducers have a systemic effect and are components of many signaling pathways, including activation of PDX1 expression.

In some cases, specific transcription factors are enhanced by direct introduction of proteins or their corresponding genes into the cell, which makes the

process more efficient. First, this concerns the PDX1 factor, which during in vivo differentiation plays an important role both at the stage of formation of pancreatic progenitors and during the maturation of β-cells [107-108].

Transplantation of cells capable of not only synthesizing and secreting insulin, but also regulating these processes depending on the concentration of glucose in the medium is an issue of fundamental importance from the point of view of the development of medical technologies.

1.6.2. Some important features of the pancreatic differentiation

Insulin-producing β-cells make up the bulk of the endocrine part of the pancreas and are part of the islets of Langerhans (60–70% of the total number of cells in the islets). Single β-cells can be located in acini and ducts [109]. β-cells maintain the basal level of insulin in the blood, ensure the rapid release of accumulated insulin and its synthesis when the level of glucose in the blood rises. In addition to insulin, β-cells secrete C-peptide into the blood in an equimolar amount - a polypeptide, after the cleavage of which insulin is formed from the proinsulin molecule. The level of C-peptide makes it possible to indirectly judge the insulin-secreting ability of β-cells, since it does not depend on insulin administered from outside [110].

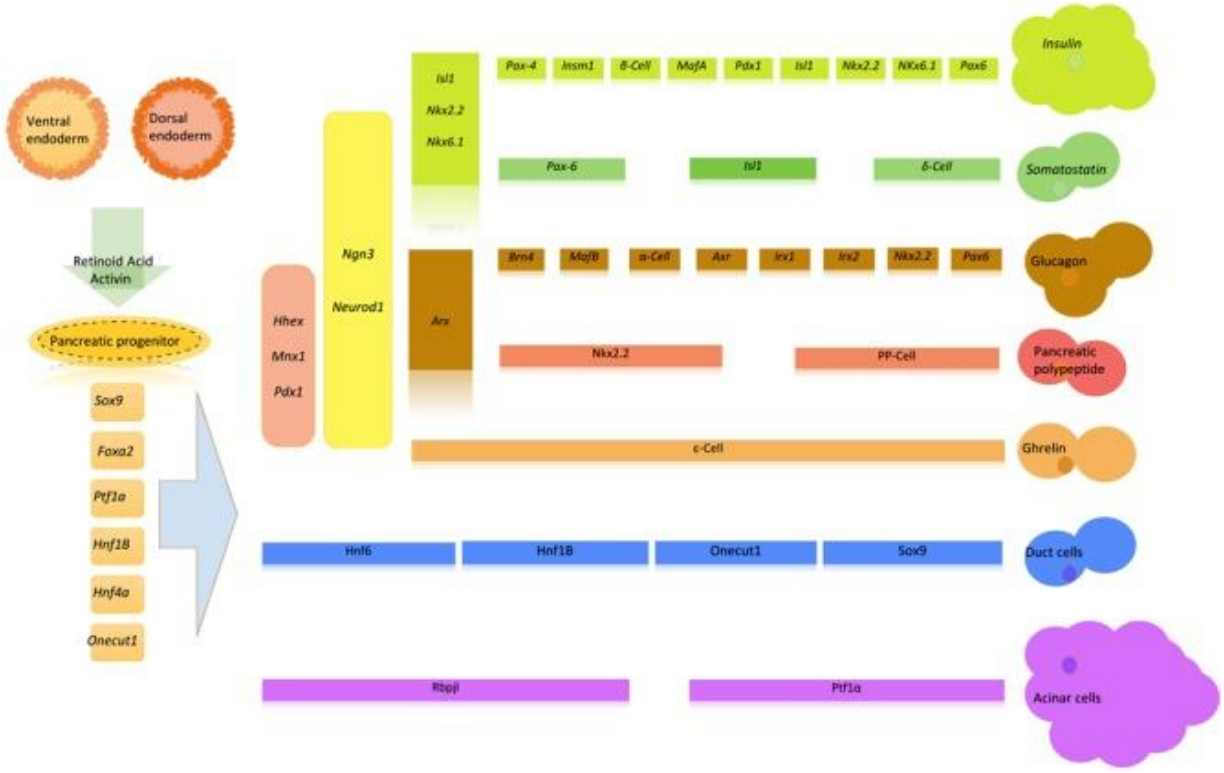

Figure 1. Schematic of pancreatic progenitors toward differentiated lineages. Upon activation of PDX1, the pancreatic fate is induced from endoderm progenitors. Pancreatic progenitors give rise to acini, ductal, and endocrine progenitors. Endocrine progenitors then differentiate into specific hormone secreting cells: α, β, δ, PP, and ε cells. Key transcription factors involved in each differentiation step and the time they are expressed are indicated [111].

β-cell differentiation has always been a complex, finely regulated process involving many signaling pathways and transcription factors (Figure 1) [111]. It should be noted that despite the desired same outcome, the detailed description of the process varies in different research groups and sources.

At the first stage of pancreatic differentiation, shortly after gastrulation and the formation of three germ layers from SCs - ectoderm, mesoderm, and endoderm - the definitive endoderm is isolated, the cells of which co-express transcription factors FOXA1 and FOXA2 [112]. The effect of other factors is also noted, for example, WNT4, FGF4, NCAD, GOOSECOID. Gastrointestinal organs are then formed from the cells of the definitive endoderm: liver, lungs, thymus, respiratory and digestive tracts, pancreas [113].

The second important stage of differentiation is the formation of a primitive intestinal tube, which gives rise to the intestine. The transcription factors PDX1 and PTF1α are involved in this process. For cells expressing PDX1 (pancreatic duodenal homeobox 1), their participation in the formation of endocrine and exocrine compartments has been proven [114]. Other factors influencing this process include FOXA4, SOX17, CXCR4, BMP2, etc. Cells of the intestinal tube with the participation of HNF1B and HNF4A differentiate into cells of the foregut, which give rise to all cells of the pancreas, including insulin-producing ones [115].

Pancreatic rudiments in the early stages of development are adjacent to the cardiac mesenchyme and notochord, which synthesize morphogens that direct the further stages of pancreatic cell differentiation: activin A, fibroblast growth factors, and bone morphogenetic factors. In the pancreatic primordia, multipotent progenitor cells are formed, which then turn into all types of pancreatic cells. For cells that are to become endocrine, the expression of PDX1, PTF1A, CPA1 and c-MYC is noted. Differentiation of endocrine cells requires inhibition of the Notch signaling pathway, which leads to increased expression of the transcription factor NGN3 (Neurogenin 3) with subsequent activation of several regulators, including NKX2.2, NKX6.1, NEUROD1, PAX4, PAX6, ISL1. The coordinated sequential action of these factors leads to IPC [116].

Overall, β-cell differentiation does not end there. At the next stages of development, a mechanism should be formed that allows cells to change the level of insulin secretion in accordance with the level of glycemia. During the maturation of β-cells, the expression of the following key factors increases:

1) INS1 (preproinsulin and insulin, which directly determine the function of β-cells);

2) GLUT2 and GK (glucose transporter 2 and glucokinase involved in the glucose sensing system);

3) PDX1, MAFA and NEUROD (transcription factors involved in the development of β-cells and their functioning);

4) CHGA, CHGB and IAPP (chromogranins and amyloid polypeptide involved in the formation of insulin secretory granules);

5) SUR1 and KIR6.1 (potassium and calcium channel genes involved in insulin secretion);

6) genes for pyruvate carboxylase, mitochondrial glycerol-3-phosphate dehydrogenase and other enzymes involved in the formation of a specific phenotype of β-cells.

In the body of an adult person, β-cells are practically not formed de novo, which leads to their rapid death during an autoimmune attack and the development of type 1 diabetes mellitus. The formation of new β-cells, both by differentiation of progenitor cells and by division of existing β-cells, has been demonstrated in adult mice and rats [117].

1.6.3. Assessment of glucose-responsivity of insulin-producing cells

Studying glucose-responsivity of insulin-producing cells is crucial for understanding and treating diabetes. This involves assessing how these cells respond to varying glucose levels, which is essential for developing effective diabetes therapies and understanding beta-cell function.

There are several methods for assessing glucose-responsive features of cells. Advanced assays using nanowell-in-microwell plates allow for the measurement of glucose-stimulated insulin secretion (GSIS) at the single-cell level. This method reveals significant heterogeneity in insulin secretion among cells, with a small percentage of cells producing the majority of insulin. Such assays are valuable for understanding the functional diversity of beta cells and stem cell-derived beta cells [118]. Insulin sensor cells use a protein-based probe to detect insulin secretion from single living pancreatic beta cells, offering a direct method to study insulin release dynamics in response to glucose [119]. C-peptide minimal model is used to quantify beta-cell function and insulin secretion in response to glucose and glucagon. They provide detailed insights into the dynamic and static control of insulin secretion, which is crucial for understanding beta-cell responsiveness in different physiological states [120,121].

Some challenges exist in terms of heterogeneity in cell response. For example, both natural and stem cell-derived beta cells exhibit significant heterogeneity in their insulin secretion response to glucose. This variability poses a challenge for developing uniform cell-based therapies for diabetes. But interestingly, the differentiation of insulin-producing cells from stem cells is influenced by glucose concentration. High glucose levels during differentiation can enhance the functionality of these cells, although it may also impair differentiation in certain cell lines [122]. As another possible approach to creating glucose-responsive cells some researchers offer developing synthetic beta cells. Research is ongoing to create artificial beta cells that mimic natural glucose-responsive insulin secretion. These synthetic cells use advanced materials and mechanisms to regulate insulin release in response to glucose levels, offering a promising avenue for diabetes treatment [123]. In addition, continued development of assays and models will enhance our understanding of diabetes pathogenesis and aid in the development of new treatments. This includes refining methods to quantify beta-cell responsiveness to various stimuli, such as glucagon and glucose [124, 125].

Indeed, the study of glucose-responsivity in insulin-producing cells is

advancing through innovative methods and models. These efforts are crucial for developing effective diabetes treatments and understanding the complex physiology of insulin secretion.

1.6.4. H1 stem cells

The H1 human embryonic stem cell (hESC) line is a widely studied model in stem cell research due to its pluripotent nature, allowing it to differentiate into various cell types. This line is used extensively in studies exploring differentiation, genetic stability, and potential therapeutic applications.

In terms of its differentiation potential, the H1 cells have shown superior efficiency in cardiomyocyte differentiation compared to other stem cell lines, such as H9 and certain induced pluripotent stem cells (iPSCs). It produces a higher abundance of cardiomyocytes, which are essential for cardiac research and potential therapies [126, 127]. In addition, H1 cells can be efficiently differentiated into neural progenitor cells using specific reporter systems, which can then be further developed into neurons. This capability is crucial for studying neurological diseases and potential treatments [128]. Among several hESC lines, H1 demonstrates a high potential for hematopoietic differentiation, making it a valuable resource for studying blood cell development and related disorders [129].

For gene expression perspectives, studies comparing H1 hESCs with isogenic iPSCs have found no significant differences in gene expression or methylation profiles, indicating that H1 maintains stable genetic characteristics under various conditions [130]. Despite its utility, the H1 line, like other hESC lines, can acquire genomic changes over time, which may include oncogenic alterations. This highlights the importance of monitoring genetic stability for safe therapeutic applications [131].

An important question in using hESCs like H1 for therapy is the risk of teratoma formation. Strategies such as inserting a suicide gene into the H1 line have been developed to selectively eliminate undifferentiated cells, enhancing safety before transplantation [132]. The H1 line is heterozygous for the ABO locus, which is crucial for avoiding immune rejection in transplantation therapies. Understanding and potentially modifying these genetic markers can improve the compatibility of stem cell-derived tissues [133].

Maintaining the pluripotency and genetic stability of H1 cells over long-term culture remains a challenge. Research continues to focus on optimizing culture conditions and genetic monitoring to ensure the safe use of these cells in clinical settings [134]. When it comes to directed differentiation, H1 cells show preferences for certain differentiation pathways, further research is needed to refine protocols for directed differentiation into specific cell types, such as chamber-specific cardiomyocytes, to enhance their therapeutic potential [127]. Although H1 stem cells have several challenges to be solved and also a few aspects to be considered while using in clinical applications, the line is still very informative and helpful in using for in vitro studies.

Therefore, the H1 embryonic stem cells is a versatile and valuable resource in

stem cell research, offering insights into differentiation processes and potential therapeutic applications, including CRISPR gene regulation systems.

1.6.5. Human somatic cell line for validation of CRISPR constructs

HEK 293 cells are a popular choice for gene regulation studies, including CRISPR, due to their versatility, ease of transfection, and ability to produce high yields of recombinant proteins. These characteristics make them particularly useful in both research and industrial applications.

There are several key advantages of using HEK 293 cells in gene regulation studies:

- *High transfection efficiency:* HEK 293 cells exhibit high transfection efficiency, which is crucial for gene regulation studies as it allows for the effective introduction of genetic material into the cells [135].

- *Versatility in protein expression:* the cells are capable of expressing a wide range of proteins, including complex membrane proteins, which is beneficial for functional characterization and studies involving protein interactions [136, 137].

- *Adaptability to different conditions:* HEK 293 cells can be adapted to various growth conditions and media, which facilitates their use in different experimental setups without the need for extensive modifications [138].

- *Gene editing and engineering:* cells are amenable to genetic modifications, such as gene knockouts and overexpression, which are essential for studying gene function and regulation. Techniques like Cas-CLOVER have been successfully used to edit genes in HEK 293 cells, demonstrating their utility in precise gene editing applications [139].

- *Transcriptomic insights:* studies have shown that HEK 293 cells can be used to explore gene expression profiles and identify potential targets for cell engineering, which can enhance the production of therapeutic vectors and proteins [140, 141].

- *Reliable and reproducible results:* The use of HEK 293 cells in gene regulation studies is supported by robust methodologies, such as qPCR, which ensure high sensitivity and specificity in detecting changes in gene transcription 142].

Thus, HEK 293 cells are an appropriate model for gene regulation studies due to their high transfection efficiency, versatility in protein expression, adaptability, and suitability for genetic modifications. These attributes, combined with reliable analytical methods, make them an invaluable tool in both basic research and the development of gene therapies. Moreover, in studies devoted to testing feasibility of various CRISPR modifications which are usually highly costly and risk-based, leveraging potential of HEK 293 cells are preferred and in times becomes an upmost priority.

2 MATERIALS AND METHODS

2.1. Research materials

2.1.1. Cell sources

HEK 293 cells were obtained from the University of British Columbia Life Sciences Institute Core Facility Center (Vancouver, BC, Canada). The obtained cells were seeded in a 6-well plate (Sigma Aldrich) and when the confluency reached to 80%, transfection process was performed on the cells. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) that was supplemented with 10% heat-inactivated fetal bovine serum (FBS, Life Technologies) and 1% penicillin/streptomycin (Life Technologies). The cells were incubated at 37ºC with 5% carbon dioxide and 95% humidity in a CO_2 incubator (Thermo Scientific, USA).

Between experiments and passages, the HEK 293 cells were stored in vapor phase liquid nitrogen below -135°C and sterile cryovials were used for storing frozen cells. HEK 293 cells are a widely used cell line in biological research and biotechnology, particularly for the production of recombinant proteins and viruses. These cells are derived from human embryonic kidney cells transformed with adenovirus DNA, which gives them unique properties that are advantageous for various applications. Therefore, due to the simplicity of cultivation and transfection, the line has become widespread in modern molecular and cell biology.

The H1 line of human embryonic stem cells (hESCs) was obtained from WiCell Research Institute, Inc. (Madison, WI, USA). The H1 cells were authenticated by Cell Line Genetics (Madison, WI, USA) and confirmed as mycoplasma-free using the MycoSEQ Mycoplasma Detection Kit (Life, Cat#4399363). Yang and others have created the H1 line [143]. Generally, H1 stem cells are widely used cell line in biomedical research. The cells were cultured in special cell culture consumables for stem cell culture (Eppendorf, Germany).

The H1 cells were maintained in a feeder-free culture system using laminin and combined with medium conditioned by mouse embryonic fibroblasts (MEF) in order to maintain the cells in undifferentiated state. The system supports the undifferentiated growth of the cells while maintaining their normal karyotype, stable proliferation rate and high telomerase activity.

HEK293T cells were obtained from the University of British Columbia Life Sciences Institute Core Facility Center (Vancouver, Canada)

2.1.2. Islets of Langerhans

The islets of human pancreas were used throughout the study for comparison reason. The islets were obtained from the Irving K. Barber Human Islet Isolation Laboratory (Vancouver, Canada), where they are typically isolated from brain-dead donors or discarded surgical specimens.

Two-Layer Method (TLM) was used for storing the islets in the lab for the study. The method combines UW solution (University of Wisconsin solution) with a perfluorochemical layer, thus allows for extended preservation times and improved

islet yields. TLM method enhances islet yields and maintain quality even after prolonged storage.

2.1.3. Plasmids

The plasmid encoding deactivated Cas9 (with H840A and D10A mutations) and VP64 that has been designed by Perez-Pinera and others [144] was obtained from Addgene (#47107). VP64 is a protein, synthetically designed as a transcription activator.

Following the algorithm that Doench J.G. and others have developed [145], several gRNAs were designed according to the sequence surrounding the transcriptional start site of *INS* gene. The plasmid pX330A_D10-1x4, which expresses native Cas9 nuclease and that was used for testing newly designed gRNAs for efficiency, was also obtained from Addgene (#58774). The gRNAs were cloned into the plasmid and the construct was then transfected into normal HEK293 cells and screened to identify the most effective gRNAs using "Surveyor" nuclease assay [146]. Eventually, the most effective two gRNAs were identified and cloned into pLV GG hUbC-dsRED plasmid, which was designed by Kabadi and others [147] and available from Addgene (#84034).

The plasmid expressing dCas9 (D10A+D839A+H840A+N863A) linked to KRAB [148], a synthetic transcription repressor domain, was also obtained from Addgene (#110820).

2.1.4. Gene blocks

During the research, numerous gene blocks, gene fragments were designed for different purposes. CRISPR technology requires many molecular modifications and alterations to be performed on genetic sequences and using relevant programs, mostly SnapGene, the sequences were created. The designed sequences were obtained from a specialized manufacturer (Integrated DNA Technologies Inc, Iowa, USA).

2.2. Research methods

2.2.1. Transfection

Transfection is a technique used for introduction of foreign nucleic acids into eukaryotic cells. The process is very essential and informative for studying gene function and expression, cellular processes, and potential therapeutic applications in practice.

In the work, transfection was performed using Lipofectamine reagent package. To transfect cells, 2-4 ug of target DNA, 5 ul of P3000 reagent and 7.5ul of Lipofectamine 3000 reagent were used for each well, according to manufacturer's instructions (Invitrogen). In total each well contained roughly 13 ul of the reaction mixture. Cells were cultured at 37^0C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Life Technologies). When 80% confluency was reached, which is roughly 1,500,000 cells in a single well of a 6-well plate, the transfection was

performed. Later, 48-72 hours post-transfection cells were collected, and total RNA was harvested for further analysis.

2.2.2. RNA isolation

Before isolating, the post-transfection cells were trypsinized (removed from the plate) and the cell pellet was washed with PBS buffer, pH 7.4 (Thermo Fisher Scientific). Next 10 ul of β-mercaptoethanol was added for each 1 ml of RLT Buffer from the RNA isolation kit.

Total RNA isolation was performed using RNeasy Mini Kit (Qiagen). Approximately 10^6 cells were harvested as a cell pellet. The appropriate volume of Buffer RLT was added, and the cells were vortexed for disruption and homogenization.

1:1 volume of 70% ethanol was added to the lysate and mixed well by pipetting (was not centrifuged). Next up to 700 µl of the sample was transferred, including any precipitate, to a RNeasy Mini spin column placed in a 2 ml collection tube and centrifuged for 15 s at ≥8000 x g. Discarded the flow-through.

On-column DNase digestion:

First, DNase I stock solution was prepared by injecting 550 µl RNase-free water into the DNase I vial using an RNase-free needle and syringe. Mixed gently by inverting the vial, no need for vortex.

Next, 350 µl Buffer RW1 was added to RNeasy column, closed the lid and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discarded flow-through. Then 10 µl DNase I stock solution was added to 70 µl Buffer RDD: mixed by gently inverting the tube and centrifuged briefly.

Following this, DNase I incubation mix (80 µl) was added directly to RNeasy column membrane, and placed on benchtop (20–30°C) for 15 min. Finally, 350 µl Buffer RW1 was added to RNeasy column, closed the lid and centrifuge for 15 s at ≥8000 x g. Discarded the flow-through.

After the on-column DNase digestion inter-step was finished, the RNA isolation was continued. Particularly, 500 µl Buffer RPE was added to the RNeasy spin column. Closed the lid and centrifuged for 15 s at ≥8000 x g, discarded the flow-through. Next, 500 µl Buffer RPE was added to the RNeasy spin column. Closed the lid and centrifuged for 2 min at ≥8000 x g.

Next placed the RNeasy spin column in a new 2 ml collection tube and centrifuged at full speed for 1 min to dry the membrane. Placed the RNeasy spin column in a new 1.5 ml collection tube and added 30–50 µl RNase-free water directly to the spin column membrane. Closed the lid and centrifuged for 1 min at ≥8000 x g to elute the RNA. At this point, the RNA isolation was completed, and spectrophotometric assessment was used for assessing the purity of the isolated RNA sample.

2.2.3. Lentivirus production

Nearly 2.5 million HEK 293T cells were plated in 10 cm plate. The next day, cells were transfected by the calcium phosphate transfection method with 5 ug of

transfer (expression) vector, 6 ug of packaging plasmid and 33 ul of Lipofectamine 3000 transfection reagent, according to manufacturer's instructions (Invitrogen). All the components were pre-diluted in serum-free Opti-MEM media (Life Technologies) before the final mix with the amount of 50 ul was added onto the cells. Then the cells were incubated at 37^0C with 5% CO_2 and the media was changed after 12–18 hours. The viral supernatant was collected 24 and 48 hours after each media change, passed through a 0.45 um filter, tittered, aliquoted (~35 ul) and stored at -80^0C for transduction procedure.

2.2.4. Transduction

HEK293 cells (with dCas9 and VP64) were plated in 12-well plate. Polybrene stock (10mg/ml) was diluted as 1/1000 in 1 ml of DMEM media (with 10% FBS and 1% penicillin/streptomycin). A total volume of pre-made lentivirus (35ul) was added to the mixture. The total mixture (DMEM + polybrene + lentivirus) was added to each well. Cells were incubated at 37^0C with 5% CO2, analyzed under fluorescent microscope (2-3 days) and flow cytometry (2 days). Next, total RNA was harvested for qPCR.

Transduction is a critical method in molecular and cellular biology, particularly in the context of gene therapy and cellular engineering. It involves the delivery of genetic material into cells using viral vectors, which can be used to modify cell function or treat diseases. In this work, transduction method was leveraged to deliver lentiviral vector with gRNAs into the test HEK 293 cells, then later into H1 stem cells.

2.2.5. Flow cytometry

48 hours post transduction cells were assayed for flow cytometry. The cells were detached from the wells using 0.5% Trypsin (Invitrogen) and pre-stained with LIVE/DEAD Fixable Near IR Dead Cell dye, according to manufacturer's instructions (Thermo Fisher Scientific). Transduced cells were gated on dsRED expressing cells. Stained cells were analyzed using a BD FACSymphony flow cytometer and FlowJo v10.3.0 software (FlowJo LLC, OR, USA). The CD45RA monoclonal antibody was used for fixing.

2.2.6. Complementary DNA synthesis and quantitative polymerase chain reaction (qPCR)

1 ug of total RNA was used for complementary DNA (cDNA) synthesis which was performed using SuperScript VILO cDNA Synthesis Kit (Invitrogen). Synthesized cDNA samples were diluted 1/10 times in ddH_2O for qPCR.

All the qPCRs were performed using PerfeCTa SYBR Green FastMix (Quanta Biosciences) in CFX96 Real-Time PCR Detection System (Bio-Rad). Data was analyzed using Sequence Detection Software (Applied Biosciences). The total reaction mixture was 20 ul in each reaction tube.

The results related to HEK 293 cells were expressed as a relative fold-increase in mRNA expression of the gene of interest normalized to housekeeping gene

(Actin) expression using the $2^{-\Delta\Delta Ct}$ method, while results of stem cells were normalized to undifferentiated H1 cells.

The qPCR results were additionally visualized using 1% agarose gel-electrophoresis.

2.2.7. Western blotting

Preparation of cells:

The cell culture was washed with sodium phosphate buffer solution (PBS), pH 7.4. Then the wash buffer was removed, and fresh PBS was added. Then a cell scraper was used to remove the cell layer. Dispensed the mixture into microcentrifuge tubes.

Next, centrifuged at 1500 rpm for 5 minutes and discarded supernatant liquid. Added 180 µl of ice-cold lysis buffer with 20 µl of fresh protease inhibitor mix. Incubated for 30 minutes on ice and then cleared the lysate by centrifuging for 10 minutes at speed 12,000 rpm at 4 °C.

Then transferred the supernatant to a clean tube and stored on ice. Measured the protein concentration using a spectrophotometer (LabTron, Inc., USA) at wavelength of 650 nm.

Preparation of samples:

Using the formula "concentration = mass/volume", determined the volume of protein extract required to place 50 µg of it in each well of the electrophoresis gel. Next placed the base and 12 mm tube monobloc on a heated stirrer (Applied Biosciences). Set the temperature to 100 °C and added 5 µl of sample buffer to each sample. All samples were well mixed. Finally, when the platform temperature reached 100 °C, warmed up the samples for 5 minutes.

Preparation of the gel:

Prepared a 10% concentration gel solution and assembled a stand for pouring the gel. Next added the concentration gel solution until the desired level is reached. Waited for 15-30 minutes for the gel to dry. Then applied the concentration gel to the separating gel and inserted the comb (must be without air bubbles), waited about 20 minutes for the gel to dry.

Electrophoresis:

Cut six filter sheets to match the size of the gel and one PVDF membrane to the same size. Moistened the gel and filter paper with blotting buffer, and the PVDF membrane - with methanol. Separated the glass plates and extracted the gel.

Next, formed a "sandwich" for the electro transfer. Placed the "sandwich" into the electro transfer device, which must be placed on ice to maintain a temperature of 4°C. Added blotting buffer to the device so that the "sandwich" is completely covered with buffer. Placed the electrodes on the "sandwich" so that the PVDF membrane is between the gel and the positive electrode. The electrophoresis lasted for 90-120 minutes in average.

Antibody blocking and incubation:

First blocked the membrane with 5% skimmed milk in tris-buffered saline with polysorbate 20 (TBST) for 1 hour. Next added primary antibody in 5% BSA

and left overnight to incubate at 4°C in a shaker. The next morning rinsed the membrane in TBST for 5 minutes (repeated this step three times overall).

Next added secondary antibody in 5% skimmed milk in TBST and incubate for one hour. Rinsed the membrane in TBST for 5 minutes (repeated three times overall). Then prepared the chemiluminescent mixture (ECL). Incubated the membrane for 1-2 minutes and visualized the result in a dark room using an ultraviolet light source.

2.2.8. Staining with Hoechst dye

Fixation of cells:

Making 4% PFA:

- Add 25ml of 1xPBS with a small magnet into a small beaker
- Place beaker on heater turned on
- Weigh 1g of PFA (stored in 4°C cold room) on rough scale in Fume Hood
- Add lg of PFA into 25ml of heated 1xPBS. Turn off heater.
- Add 2 drops of NaOH
- NaOH should dissolve, otherwise turn on heater for a while. Cool to Room temperature

On bench:

- Aspirate media of the cells in wells and wash with PBS
- Wash cells 1xPBS
- Apply 4% PFA (Room temperature) for 20min (500ul)
- Wash cells 1xPBS
- Apply 0.1% TritonX (Permeabilization) (500ul) for 15min
- Wash cells 1Xpbs

Primary antibody staining:

- Add wet tissue paper in the dark humid chamber and place pieces of broad parafilm hereon
- Aspirate TritonX away
- Wash cells 1xPBS
- Add 30ul of protein blocker on parafilm (DAKO, Ref X0909, Lot 10084945)
- Place coverslips on parafilm. Cells should be down on blocking buffer!
- Incubate cells in blocking buffer for 1h (dark) at Room temperature
- (Prepare primary antibody solutions, diluted in DAKO, Ref S0809, Lot 10089538)
- Add coverslips back in 24 well plate. Cells should face up!
- Wash 2x with PBS in well plate
- Place 30ul drops of primary AB on new parafilm in humid chamber
- Place Cells (cells facing down on primary AB solution) overnight at 4°C.

Secondary antibody staining:

- (Start this process in the morning, so they are ready to image the next day)
- Add coverslips back into 24-well plate - Surface with cells should be up.
- Wash with 1xPBS and aspirate

- Wash and cast for 6x10min in 1xPBS (300 rpm in mini shaker) at Room temperature while shaking
- Meanwhile prepare secondary antibodies (diluted in DAKO, Ref S0809, Lot 10089538)
- Spin down secondary antibodies at max speed for 10 min (to get crystals stuck at the bottom)
- Place 30ul drops of secondary antibodies on parafilm in humid chamber at room temperature for 90 min.
- Wash 10x10 min in 1xPBS. Take out mounting media from -25°C. Keep in dark!
- During the last wash prepare Hoechst staining dilution for the next step:
- Wash glass slides with ethanol, wipe clean. Let dry, so they are ready for mounting.

Hoechst staining and mounting:

- Was and aspirate away 1xPBS
- Incubate cells with 1:10 000 dilution of Hoechst staining (500ul) (33342, Molecular Probes, Lot 38231A, stored at 4°C) for 7 min (1:10 000 Hoechst dilution can be used for 3 months while kept in dark)
- Wash with 1xPBS
- Rinse coverslip in H_2O (dip into container with ultrapure H_20) + dry (dip on blotting paper)!
- Add 9-10 ul of mounting media (no bubbles!) on slide with Prolong Gold anti fade reagent (P36930, Lot:118942) (mounting media) (Warmed to Room temperature)
- Add dried coverslips. Cells should face down!
- Let it sit overnight on bench (Room temperature)
- Image the following day.

2.2.9. Designing guide RNAs

The guide RNAs were designed using GuideMaker tool [149]. The tool is a software solution designed to facilitate the creation of CRISPR-Cas guide RNA (gRNA) pools, particularly for non-model genomes and non-standard Cas enzymes. The tool addresses the need for efficient gRNA design in complex and less-studied organisms, expanding the potential for gene editing beyond traditional model organisms.

2.2.10. Delivery of the target constructs into H1 stem cells

At this step, the activation construct and the lentiviral constructs were delivered into the H1 stem cells through transfection and transduction methods, respectively. Overall, all the procedures, performed with HEK 293 cells, were accordingly repeated for the H1 stem cells.

2.2.11. *In vitro* differentiation of H1 stem cells to pancreatic β-cells

H1 stem cells culture:

H1 cells were cultured on 1:30 diluted Matrigel (BD BioSciences, CA, USA) in Essential 8 (E8) medium (Life Technologies, USA). When the confluency reached 70–80%, cultures were rinsed with 1× DPBS without Mg2+ and Ca2+ (Invitrogen, USA) and were incubated with TrypLE Express Enzyme (1×) (Life Technologies, USA) for 3–5 min at 37 °C. This leads to some single cells to be detached, and the released cells were rinsed with E8, spun at 1,000 rpm for 5 min. Next the pellet was washed with E8 medium supplemented with Y-27632 (10 mM; Sigma-Aldrich; MO, USA). The resulting cell suspension was seeded at 1.3–1.5 × 10^5 cells per cm^2 on Matrigel-coated surface. The culture was kept in E8 medium, and the differentiation process started after 48 hours of seeding with confluency around 90%.

The overall differentiation process of human stem cells to insulin producing β-cells consisted of 7 stages with overall duration of 27 to 43 days depending on how quickly the needed cells were reached on each step.

Stage 1: Definitive endoderm:

The duration of this first stage was 3 days. The H1 stem cells were first plated on 1:30 Matrigel-coated surface, then rinsed with 1× DPBS without Mg2+ and Ca2+ and exposed to MCDB 131 medium (Life Technologies, USA) supplemented with 1.5 g/l sodium bicarbonate (Sigma, MO, Cat# S6297), 1× Glutamax (Life, Cat#35050-079), 10 mM final glucose (Sigma, Cat# G8769) concentration, 0.5% BSA (fatty acid free BSA, Proliant, IA, Cat#68700), 100 ng/ml GDF8 (Pepro-Tech; Rocky Hill, NJ, Cat#120-00), and 1 mM of MCX-928 (GSK3b inhibitor3) for day 1 only. For day 2, cells were cultured in MCDB with 0.5% BSA, 1.5 g/l sodium bicarbonate, 1×Glutamax, 10 mM glucose, 100 ng/ml GDF8 and 0.1 mM of MCX-928. On day 3, cells were cultured in MCDB with 0.5% BSA, 1.5 g/l sodium bicarbonate, 1× Glutamax, 10 mM glucose and 100 ng/ml GDF8. This order resulted in almost 100% expression of key FOXA2 and CXCR4 markers by the cells.

Stage 2: Primitive gut tube:

This stage lasts 2 days and the goal is to keep the expression of FOXA2 and stop CXCR4's expression. The cells of stage 1 were rinsed with 1X DPBS (without Mg2+ and Ca2+) and exposed to MCDB 131 medium supplemented with 1.5 g/l sodium bicarbonate, 1XGlutamax, 10 mM final glucose concentration, 0.5% BSA, 0.25 mM ascorbic acid (Sigma, Cat# A4544) and 50 ng/ml of FGF7 (R&D Systems, Cat#251-KG) for 2 days.

Stage 3: Posterior foregut:

The cells from stage 2 were incubated in BLAR medium supplemented with 2.5 g/l sodium bicarbonate, 1× Glutamax, 10 mM glucose, 2% BSA, 0.25 mM ascorbic acid, 50 ng/ml of FGF7, 0.25 mM SANT-1 (Sigma, Cat# S4572), 1 mM retinoic acid (RA; Sigma, Cat#R2625), 100 nM LDN193189 (LDN; BMP receptor inhibitor, Stemgent, CA, Cat#04-0019), 1:200 ITS-X (Life, Cat#51500056), and 200 nM TPB (PKC activator, custom synthesis, ChemPartner, China) for full 2 days. At the end of this stage the cells should express key PDX1 protein marker.

Stage 4: Pancreatic endoderm:

By the end of this stage, which lasted for 3 days, the cells were expected to express both PDX1 and NKX6.1 markers. Overall, the stage 3 cells were further

incubated in the BLAR medium which contained 2.5 g/l sodium bicarbonate, 1×Glutamax, 10 mM glucose, 2% BSA, 0.25 mM ascorbic acid, 2 ng/ml of FGF7, 0.25 mM SANT-1, 0.1 mM retinoic acid, 200 nM LDN193189, 1:200 ITS-X and 100 nM TPB for 3 days. After day 3 the cells were treated for 4 h with 10 mM Y-27632. Cells were then rinsed with 1× DPBS without Mg2+ and Ca2+ and then exposed to TrypLE (1×) for 3–5 min at room temperature. The released cells were washed with basal BLAR medium and spun at 1,000 rpm for 3 min. The resulting cell pellet was resuspended as single cells at a density of ~0.5 × 105 cells/ml on filter inserts (BD, Cat#35-3493 or Corning Cat#3420); 5–10 ml per spot for a total of 0.25–0.5 × 106 cells/spot) at an air-liquid interface. Each spotted area measured ~1–2 mm in diameter depending on the volume of cells added. For 6-well filter inserts (BD), 1.5 ml/well was added to the bottom of each insert whereas 8 ml was added for 10-cm filter inserts (Corning). Typically, 10–15 spots were used per well of a 6-well insert and 80–90 spots were used for 10-cm inserts.

Stage 5: Pancreatic endocrine precursors:

Key markers the cells are expected to express at this stage are PDX1, NKX6.1 and NEUROD1. The obtained stage 4 cells within next 3 days were cultured in BLAR medium supplemented with 1.5 g/l sodium bicarbonate, 1× Glutamax, 20 mM glucose, 2% BSA, 0.25 mM SANT-1, 0.05 mM retinoic acid, 100 nM LDN193189, 1:200 ITS-X, 1 mM T3 (3,3′,5-Triiodo-l-thyronine sodium salt, Sigma, T6397), 10 mM ALK5 inhibitor II (Enzo Life Sciences, NY, Cat# ALX-270-445), 10 mM zinc
sulfate (Sigma, Z0251) and 10 mg/ml of heparin (Sigma, H3149). When heparin was added to culture it provided more viability of clusters of cells.

Stage 6: Cells expressing NKX6.1 and insulin:

(9–12 d). The cells of stage 5 were further cultured in BLAR medium supplemented with 1.5 g/l sodium bicarbonate, 1× Glutamax, 20 mM glucose, 2% BSA, 100 nM LDN193189, 1:200 ITS-X, 1 mM T3, 10 mM ALK5 inhibitor II, 10 mM zinc sulfate, 100 nM gamma secretase inhibitor XX for 7 days (EMD MilliPore, MA, Cat# 565789) and 10 mg/ml of heparin for 9–12 days.

Stage 7: Cells expressing NKX6.1, insulin and MAFA marker:

Stage 6 cells were exposed to BLAR medium supplemented with 1.5 g/l sodium bicarbonate, 1× Glutamax, 20 mM glucose, 2% BSA, 1:200 ITS-X, 1 mM T3, 10 mM ALK5 inhibitor II, 10 mM zinc sulfate, 1 mM N-acetyl cysteine (N-Cys, Sigma, Cat# A9165), 10 mM Trolox (Vitamin E analogue, EMD, Cat#648471), 2 mM R428 (AXL inhibitor, SelleckChem, Cat# S2841) and 10 mg/ml of heparin for 12 days. During all stages the culture's medium was changed every day.

2.2.12. Statistical analysis of data

Statistical analysis was performed using GraphPad Prism 8.0 software (GraphPad Software Inc, CA, USA). Data are presented as bars and dot plots with mean values ± standard deviation. The data were evaluated by one-way ANOVA analysis of variance accounting for different variances across the groups, with post-hoc Tukey's or Sidak's multiple comparisons test. Statistical significance is

represented as *p<0.05, ** p<0.01, *** p<0.001 or **** p<0.0001.

2.2.13. Testing glucose-responsive feature of insulin producing cells

To determine glucose-responsive feature of the differentiated cells 3 concentration of glucose was used: 5 mM, 15mM and 25mM. Cells were first incubated for 30 minutes at 5 mM glucose for equilibration. Then 5mM, 15mM and 25mM of glucose was used to incubate the cells for 120 minutes in total. After the time past, the media was collected and stored at -80 ^{0}C. The insulin produced by the cells into the media was determined using a rat/mouse insulin ELISA kit (Merck Millipore) following the manufacturer's instructions. The level of insulin produced and respective change in the protein overall concentration in the media was determined using Bradford assay (Bio-Rad, USA).

2.2.14. Protein quantification using Bradford assay

5 μL sample of cell lysate from each well was moved to a 96-well assay plate. Next, 200 μL Bradford reagent was added to each well and the mixture was incubated for 10 minutes. Then the absorbance was measured using a spectrophotometer (LabTron, Inc., USA) at wavelength of 570 nm and the protein content values extracted from the standard curve.

3 RESULTS AND DISCUSSION

Application of CRISPR technology to genome of stem cells first needs to be tested and validated in ordinary human cells for known reasons. Firstly, studies on stem cells are usually laborious and time-consuming. Therefore, it is always a good logic to do necessary modifications and applications in human test cells to confirm the technology so that when it gets to the stage of stem cells the procedure is well-planned, managed and precisely performed. Secondly, in general, studies involving any type of stem cells are much costly than with any other ordinary human cells and therefore well-planned research budget has always been important in achieving desired study outcome.

Studying the feasibility of modulating insulin transcription in stem cells with CRISPR technology requires to first design the constructs and then test them accordingly in ordinary human cells. For this reason, a well-known HEK293 (human embryonic kidney-293) cells were used.

3.1. Validation of the constructs in HEK 293 cells

3.1.1. Obtaining dCas9-VP64 HEK 293 cells

Testing the proposed technology first began with obtaining a stable line of HEK 293 cells that would stably express the deactivated form of the Cas9 nuclease (dCas9) and well-known transcription activator VP64 protein. As there was no need to cleave the target DNA in the experiments, dCas9 was used instead of natural Cas9. In the full gene regulation construct VP64 domain, linked to the nuclease, acts as an activator of transcription when brought close to the promoter of an endogenous gene. As stated above, the dCas9 nuclease has been designed earlier by Jinek et al. via inducing point mutations into the catalytic amino acid residues (D10A, H840A) of natural Cas9.

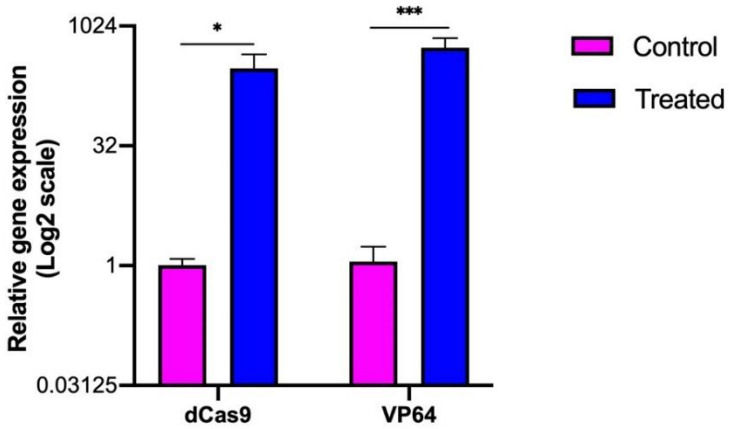

Figure 2. Obtained stable cell line of HEK 293 cells expressing dCas9 and VP64 domains. Transfected cells (treated) showed relatively high expression of both proteins compared to control (non-transfected) cells. Statistical analysis was performed by a two-way ANOVA test followed by Sidak's post-hoc test for multiple comparisons [152].

The HEK 293 cells were transfected with a plasmid encoded with both the dCas9 and the VP64 where both proteins are linked together. The expressions of the target proteins were measured, and the experiment was continued until sufficient and stable expressions of the proteins were achieved in the transfected cells (Figure 2).

The gene expression measurements were performed using qPCR. The ultimate primer concentration for each studied gene mixture was 200 nM and the sequences of the primers for each gene was the following:

dCas9 forward: AATACCGATCGCCACAGCAT;
dCas9 reverse: CCTGTATTAGCCATGGAACA;
VP64 forward: ATCAGCTTGTCGCTGTTCCT;
VP64 reverse: CGGTTATTCCATCGTAATTC.

3.1.2. Preparation and cloning of guide RNAs

Designing guide RNAs (gRNAs) for CRISPR gene regulation constructs is a critical aspect of leveraging CRISPR technology for precise genome editing and transcriptional control. The process involves creating a library of synthetically designed gRNAs, selecting out the most efficient ones and also possibly optimizing the sequences to ensure specificity and efficiency in targeting desired genomic sequence.

In order to design gRNA for CRISPR technology, expectedly, nucleic acid sequence of the target sequence needs to be studied and there are a number of online tools for that. In this research promoter of insulin gene acted as target.

It has been experimentally shown earlier that synergistic effect of several gRNAs (in some cases up to 4) targeted to the same transcriptional site significantly enhance the overall efficiency of endogenous gene activation compared to when a single gRNA is used [150]. Taking this into account and following the algorithm that has been described above, a number of INS gRNAs were designed using GuideMaker tool. Next, using the Surveyor nuclease assay the most effective gRNAs from the library was selected. In the assay the natural Cas9 nuclease is used so that the effectiveness of the gRNA is assessed based on the successful cleavage of the target sequence by the enzyme. Thus, after several rounds of the assay run, 2 gRNAs were selected the sequences of which were as follows:

INS gRNA 1: AACTCCTCGTGAAGATGCCC
INS gRNA 2: ATGGTCATCAAGCGACCTGG.

After identifying the most effective gRNAs targeted to insulin promotor, the next step was to clone them into a vector for delivering into host cells. Therefore, in the research, the gRNAs were delivered to cells of interest (first HEK 293 cells and next stem cells) separately from the CRISPR sequence. For the delivery a plasmid vector with a fluorescence reporter gene was chosen - pLV GG hUbC-dsRED plasmid deposited to Addgene database earlier by another research team. The selected gRNAs were cloned into the pLV GG hUbC-dsRED plasmid where gRNA 1 was under control of hU6 promotor and the gRNA 2 was driven by mU6. The total size of the resulted plasmid was 10766 base pairs (Figure 3).

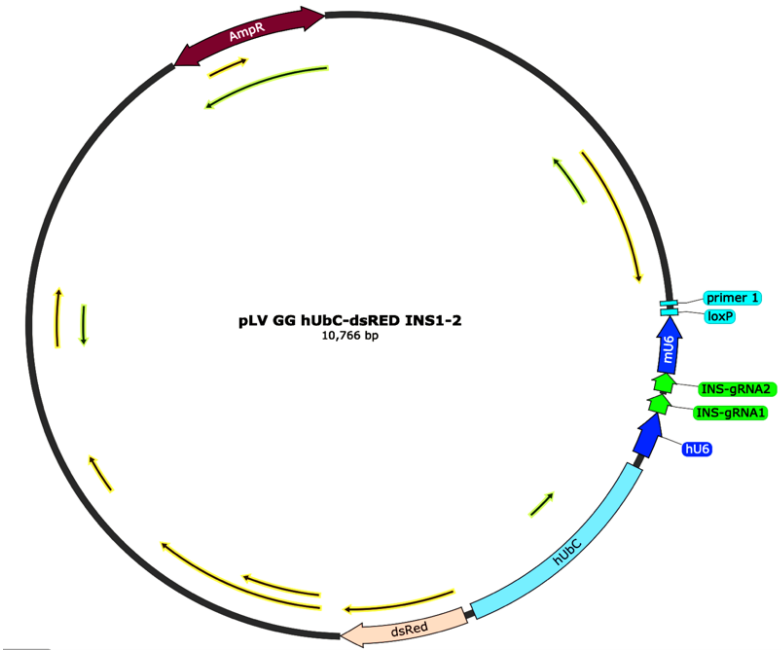

Figure 3. View of the pLV GG hUbC-dsRED plasmid with 2 *INS* gRNAs cloned in. Each *INS* gRNA is driven by separate promoters, dsRED is a reporter gene (driven by hUbC promoter).

The human ubiquitin C (hUbC) promoter is known for its ability to drive consistent and sustained expression of transgenes across different cell types and developmental stages. In the context of cloning this characteristic is crucial for the successful development of transgenic cell lines. In this work, human UbC promoter was used to drive the expression of the reporter gene dsRED as a part of the insulin gRNAs-expressing plasmid.

3.1.2.1. Plasmids and promoters for cloning

Apart from the plasmids used for gRNA preparation and cloning, there were two other plasmids used for the research. The mentioned above dCas9-VP64 HEK 293 cells were obtained by transfecting ordinary HEK 293 cells with the plasmid encoding dCas9 (with) and VP64 activator. The dCas9 nuclease has been obtained by introducing point mutations of H840A and D10A into the catalytic site of the native Cas9 nuclease. The dCas9-VP64 plasmid was purchased from a well-known plasmid repository Addgene by the number 47107. The plasmid is useful for using in mammalian expression experiments, including CRISPR.

The plasmid expressing another version of dCas9 nuclease (D10A+D839A+H840A+N863A) and which is linked to KRAB transcription repressor was also purchased from Addgene (available by #110820).

Both the dCas9-VP64 and dCas9-KRAB plasmids contained a resistance gene against ampicillin which made it possible to select respective colonies during growth of bacteria containing the plasmids.

In regard to promoters, CMV promoter drove the expression of the nuclease and the respective synthetic transcription factor in both of the dCas9-VP64 and dCas9-KRAB plasmids. The CMV promoter (human cytomegalovirus) was a sequence of 204 base pairs in both plasmids. The promoter exhibits strong and constitutive activity and thus is widely used in molecular biology for driving the expression of transgenes in eukaryotic cells. The CMV promoter is also extensively used in gene therapy and recombinant protein production.

Another promoter used in the work was human U6 promoter (hU6) which was used to drive the expression of insulin gRNA 1 in the pLV GG hUbC-dsRED plasmid with 2 insulin gRNAs. Indeed, the hU6 promoter is a widely utilized tool in molecular biology for driving the expression of short hairpin RNAs (shRNAs) and small interfering RNAs (siRNAs) in mammalian cells. This promoter is also integral to RNA interference (RNAi) technologies, which are used for gene silencing and functional genomics studies. The promoter is also used in combination with other elements to create hybrid systems for versatile applications in gene editing [151].

The mU6 promoter, which was implemented to drive the expression of the second insulin gRNA, is in fact a key element in gene editing and gene silencing technologies, particularly within the CRISPR/Cas9 system and RNA interference methods. The promoter is a type of RNA polymerase III promoter commonly used to drive the expression of small guide RNAs (sgRNAs) and short hairpin RNAs (shRNAs), which are crucial for the efficiency of these genetic tools.

Both the hU6 and mU6 promoters were selectively used to drive the expressions of gRNAs in the respective construct and showed relatively high performance.

3.1.3. Delivery of the gRNAs into HEK 293 cells

After the gRNA-expressing plasmid was obtained, next it was packaged into lentiviral vector using lentivirus production method described earlier. Obtained concentrated viral material was then used to infect the previously produced dCas9-VP64 HEK 293 cells.

The idea of delivering lentiviral material with gRNAs into the host cells which already expresses other needed genes of the CRISPR system was done on two important research reasons. Firstly, experience of previous authors shows that in manipulating insulin gene with genome editing tools it is hard to get effective result if to deliver all the CRISPR complex in one vector. Secondly, and consequently of the first reason, it is preferable and more effective to deliver gRNA alone into host cells when the host cells are already stably expressing the other genes. Therefore, considering all these being said, we have decided to infect the HEK 293 cells separately with lentivector using well known transduction method.

Thus, the procedure of transduction was executed, and the result of the infection first was tested using flow cytometry. Quantitative PCR method and fluorescence microscopy methods were also considered in case of any unclear outcomes with the cytometry or only to confirm the results.

The flow cytometry showed that lentiviral gene construct encoded with insulin

gRNA was successfully transduced with 43.7% efficiency into the host cells (Figure 4, part C).

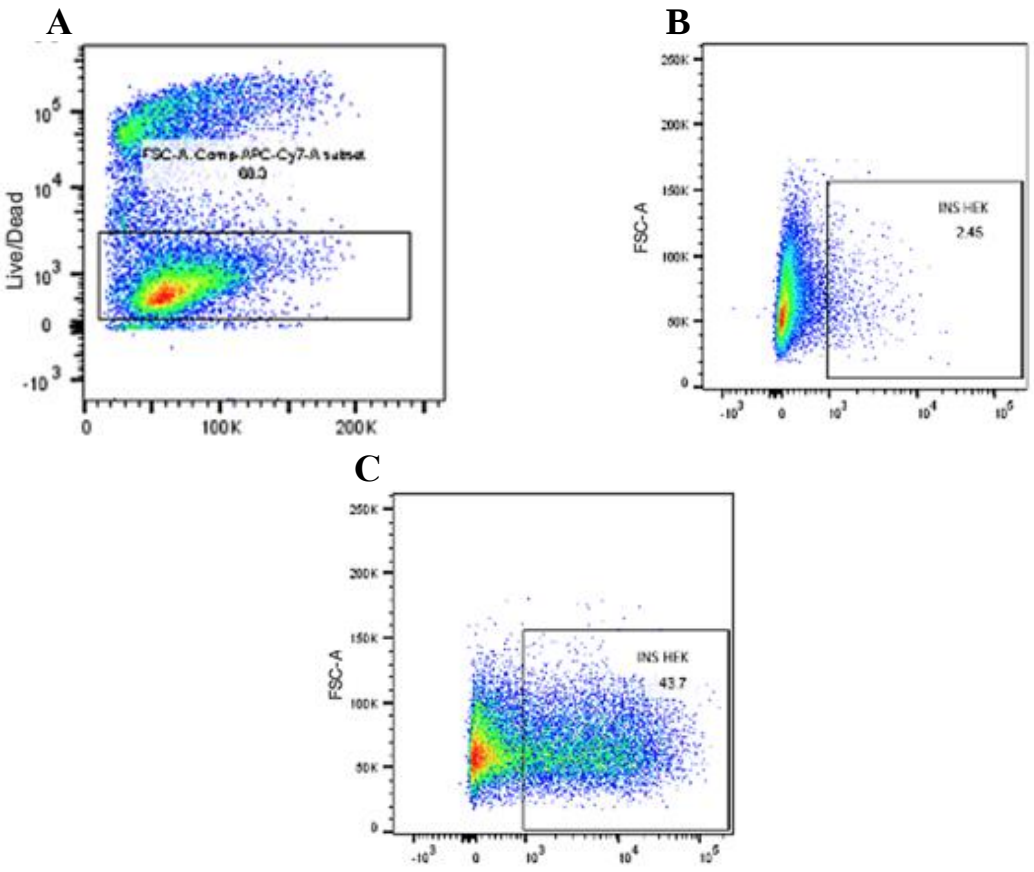

Figure 4. Transduction of dCas9-VP64 HEK 293 cells with Lenti *INS* gRNA: **A** – Overall flow cytometry result of the transduced cells; **B** – unspecific transduction; **C** - successful transduction with 43.7% efficiency. LIVE/DEAD Fixable Near IR Dead Cell dye LIVE/DEAD Fixable Near IR Dead Cell dye (Thermo Fisher Scientific) was used with CD45RA monoclonal antibody [152].

For purposes of detecting directly and more specifically binding, CD45RA single antibody was used. For the away, 48 hours post transduction cells were used. The cells were detached from the wells using 0.5% trypsin protease and pre-stained with LIVE/DEAD Fixable Near IR Dead Cell dye (Thermo Fisher Scientific). All the transduced cells were gated on dsRED expressing cells, pre-cloned reporter in the lentiviral vector. The assay results were analyzed using BD FACSymphony flow cytometer machine and FlowJo v10.3.0 software.

The antibody's non-specific binding result was equal to 2.45% which is quite high specificity. Meanwhile the specificity was 43.7%.

On top of the flow cytometry outcome the fluorescent microscopy was also leveraged which confirmed adequately successful integration of the pLV GG hUbC-dsRED plasmid genes into the host cells demonstrating expression of the reporter gene (dsRED, Figure 5).

As a result of the infection, mutant HEK 293 cells started to express all the necessary proteins for targeting and activating the transcription of insulin gene. This becomes possible due to gRNA rendering dCas9-VP64 complex to a close proximity to the promotor of the target gene due to gRNA's complementarity to the promotor.

gRNA and dCas9 nuclease interact in a highly coordinated manner to enable precise DNA targeting in the CRISPR-Cas9 system. In nature, the binding of gRNA to Cas9 induces a conformational change in the Cas9 protein, transitioning it from an inactive state to an active form. This structural rearrangement is crucial for DNA recognition and cleavage. In the case of dCas9, interaction with gRNAs stabilizes the nuclease's form, but do not activate it due to the fact that the nuclease is already inactive and facilitates the transcription activator VP64 interact with the insulin promotor.

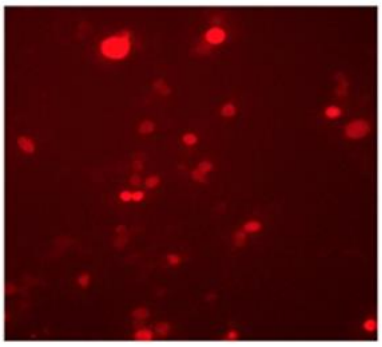

Figure 5. Transduction of dCas9-VP64 HEK 293 cells with Lenti *INS* gRNA. Representative image of successfully transduced cells shown in red captured by fluorescent microscopy. dsRED acted as fluorescent reporter. Zoomed using EVOS M5000 imaging system with EVOS™ 40X lenses [152].

3.1.4. Insulin transcription activation in HEK 293 cells

After obtaining respectively adequate expressions of the needed genes, the next and most responsible step was to check if the CRISPR construct achieved insulin transcription activation. The analysis was done using quantitative polymerase chain reaction (qPCR). This real-time qPCR is widely used for measuring gene expression levels due to its robustness and reliability. It has largely replaced older methods such as Northern blotting and RNase protection assays and has become a routine approach for validating microarray experiments and monitoring biomarkers. In fact, the method is applicable across various fields, including basic molecular biology, medicine, and diagnostics, allowing for the comparison of mRNA levels in different biological samples.

In the research the qPCR showed that the inserted CRISPR genes properly expressed and collectively completed their function as shown by significant fold increase (over 900x) in ultimate insulin transcription level in test samples compared with negative controls (Figure 6). Samples with no addition of insulin gRNAs were regarded as the negative controls, no gRNA – no targeting of the insulin promotor.

In addition, comparison was made between the studies test samples and insulin-producing Min6 cells obtained from mouse. Level of activated insulin transcription level in test samples was roughly 5x times less than in the Min6 cells.

Min6 cells are a pancreatic beta-cell line derived from insulinomas in transgenic mice, widely used for studying insulin secretion and beta-cell function. The cells are valuable for understanding the molecular mechanisms of insulin regulation and the effects of various stimuli on beta-cell activity. Min6 ells exhibit glucose-inducible insulin secretion similar to normal mouse islet cells. They respond to increasing glucose concentrations with a progressive increase in insulin secretion, reaching a maximum at higher glucose levels. This also makes them an appropriate model for studying glucose-stimulated insulin secretion. The glucose metabolism in MIN6 cells can be characterized by rapid glucose transport and phosphorylation, primarily through glucokinase, which constitutes the majority of phosphorylating activity. This reflects the glucose utilization patterns seen in normal islets [153].

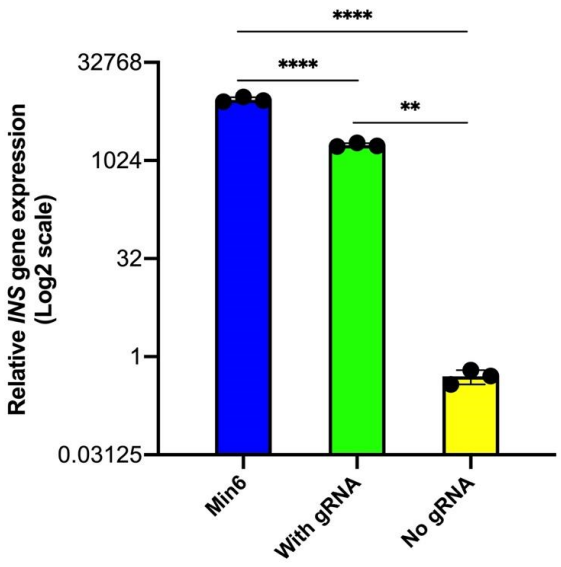

Figure 6. HEK 293 cells *INS* gene activation with gRNA-directed VP64 transcription factor fused to dCas9: Min6 – insulin-producing mouse cell line (positive control); With gRNA – dCas9-VP64 HEK 293 cells with activated *INS* gene; No gRNA – only dCas9-VP64 HEK 293 cells. Statistical analysis was performed by a one-way ANOVA test followed by Tukey's post-hoc test for multiple comparisons. *P < 0.05; **P < 0.01; ***P < 0.001; ****P<0.0001 [152].

For the qPCR analysis of the insulin transcription activation in HEK 293 cells the following pair of primers were leveraged:

Forward INS primer: 5'-AAGTTGTCGACAGGCTGCATCAGAAG-3'
Reverse INS primer: 5'-ATAGGATCCACAGGGACTCCATCAG-3'.

The activated insulin gene expression in HEK 293 cells was additionally confirmed by staining. As described above, the construct with insulin gRNA also

contained a dsRED reporter gene providing us an opportunity to visualize the activated target gene. The fluorescence activity of the reporter was observed with a respective fluorescent microscope and imaged accordingly (Figure 11). For detecting the target product rabbit insulin antibody was used.

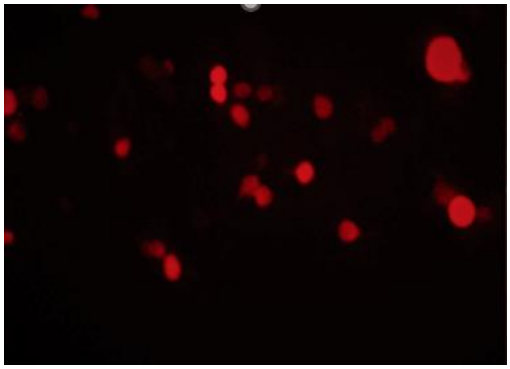

Figure 11. Fluorescent image of the HEK 293 cells with successful activation of insulin's transfection. Zoomed using EVOS M5000 imaging system with EVOS™ 40X lenses.

3.1.5. Insulin transcription repression in HEK 293 cells

After successful activation of the insulin transcription, we next aimed to check if it would be feasible to further manipulate the gene. Particularly, the goal was to try to lower the recently activated *INS* transcription. Several studies have previously showed that Kruppel-associated box (KRAB) protein could be as a strong transcription inhibitor for most endogenous genes. The KRAB domain functions as a transcriptional repressor by binding to corepressor proteins, primarily KAP1 (KRAB-associated protein 1). This interaction is essential for the repression activity, as KAP1 enhances KRAB-mediated repression and can act as a repressor when tethered to DNA. The KRAB domain's repressive function is dependent on its ability to recruit KAP1, which then facilitates the deposition of repressive histone marks such as H3K9me3, leading to heterochromatin formation and transcriptional silencing. The KRAB domain is a conserved 75-amino acid region containing amphipathic alpha-helices necessary for its repressive function. Mutations in these helices can abolish repression, highlighting their importance in the KRAB domain's activity [154].

Thus, the HEK 293 cells with activated insulin gene (dCas9-VP64+ Lenti *INS* gRNA) were transfected with the previously obtained plasmid with dCas9-KRAB complex. This dCas9 had a slightly different amino acid sequence than the previous one in dCas9-VP64 but can bind with the *INS* gRNAs which are still expressed by the host HEK 293 cells.

As a result of the transfection, substantially lower amount of insulin mRNA (nearly 600x) was observed in the infected cells compared to control samples (no dCas9-KRAB addition, Figure 7) [152].

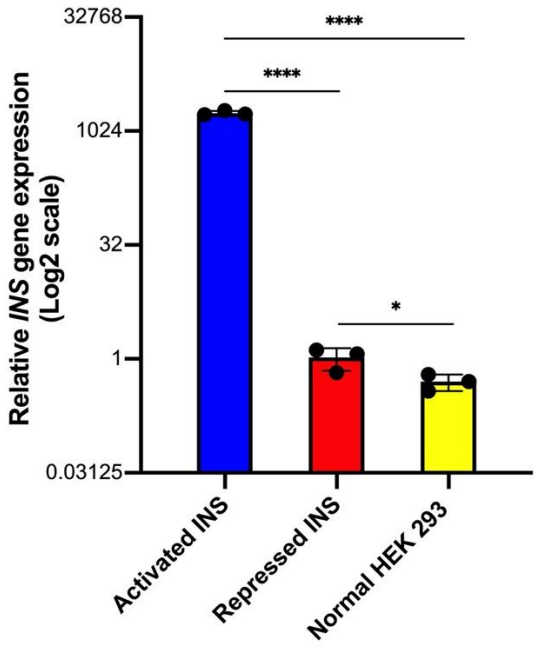

Figure 7. Repression of the recently activated *INS* gene in dCas9-VP64 HEK 293 cells: Activated INS – positive control; Repressed INS - [gRNA + (dCas9-VP64)] + (dCas9-KRAB); Normal HEK 293 cells - negative control. Statistical analysis was performed by a one-way ANOVA test followed by Tukey's post-hoc test for multiple comparisons. *P < 0.05; **P < 0.01; ***P < 0.001; ****P<0.0001

Lowering insulin expression was achieved due to expression of the genes of the repression construct. Respectively, when qPCR was completed, the transfected cells showed highly expressed dCas9 and KRAB levels (Figure 8).

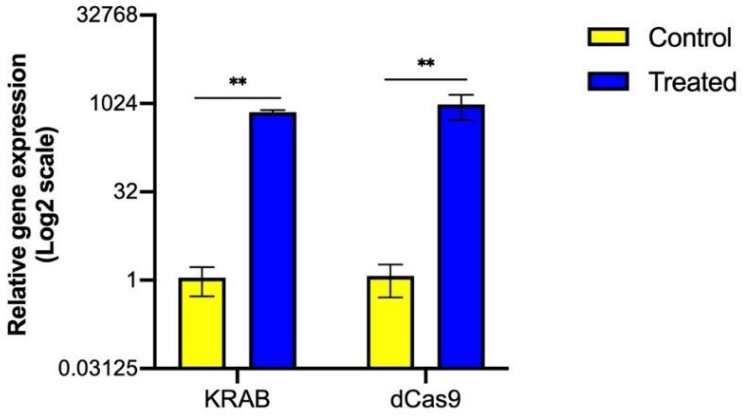

Figure 8. Expressions of repressor protein domains in (dCas9-VP64+*INS* gRNAs) HEK 293 cells transfected with dCas9-KRAB plasmid (#110820). As seen, both proteins expressed well which led them act together as *INS* gene transcription repressors in complex with gRNAs. Statistical analysis was performed by a two-way ANOVA test followed by Sidak's post-hoc test for multiple comparisons. *P < 0.05; **P < 0.01; ***P < 0.001; ****P<0.0001.

The expression of the transfected domains was analyzed using qPCR method as above described for which the following primers were utilized (the primer for dCas9 was slightly different from the primer for the dCas9-VP64):

dCas9 forward: AGTCCCGATCGCTTCAGCAG;
dCas9 reverse: CCTTCATTAGTTATGCCACA;
KRAB forward: ATATTTTGTTCCTCAGCCGC;
KRAB reverse: ATCGGTTGTAATCATATCTT.

It is worth noting that the repression experiment was leveraged only to check feasibility of manipulating already activated gene expression. The study was not related to the main continuation of the whole research which is to achieve the target gene transcription activation in stem cells. Importantly, the repression experiment shed additional light on possibilities of modifying endogenous insulin expression using artificial transcription factor linked to dCas9 nuclease. We understood that adding different and more dCas9 to the media can cause competition for the existing dCas9 (linked with VP64) to be integrated with some of the gRNA. And also, as noted above and proved by some scientific data, having insulin production repressed at certain points of the disease development may be beneficial for overall outcome of the disease management.

3.1.6. Western blotting

Majority of genes analyzed with quantitative polymerase chain reaction (qPCR) method was followed by Western blotting for protein analysis test. In transcription regulation studies it is a big plus if needed genes' expression along with mRNA level is also detected and analyzed on protein level.

In the research, western blotting was utilized as a backup method to confirm needed genes' expression depending on availability of necessary antibodies. Below can be found some of blotting images performed on HEK 293 on various stages of the project. Due to availability of rabbit VP64 and dCas9 antibodies, the respected proteins were chosen to be detected with blotting (Figures 9 and 10).

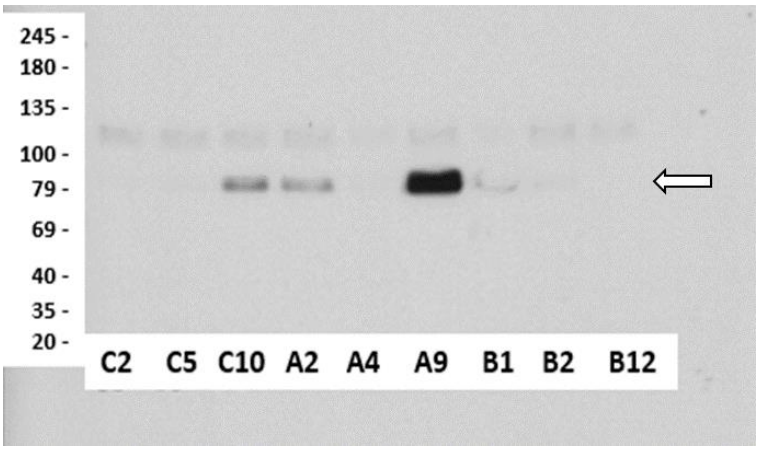

Figure 9. Western blot image of VP64 expression in HEK 293 cells. Expected molecular weight is 81 kDa, detected by VP64 rabbit antibody.

For the blotting analysis, 50 μg of protein extract was used for electrophoresis and 10% concentration gel was prepared. Up to 9 samples were used and the samples with the best result was kept for further stages of the research. As noted above, the blotting was done for those proteins for which respective antibodies were in the laboratory. Usually, in gene regulation studies western blotting is not leveraged as extra confirmation method unless qPCR or other main and less laborious techniques are demonstrating not adequate results. Indeed, Western blotting is time-consuming and laborious compared to other modern protein-analyzing methods.

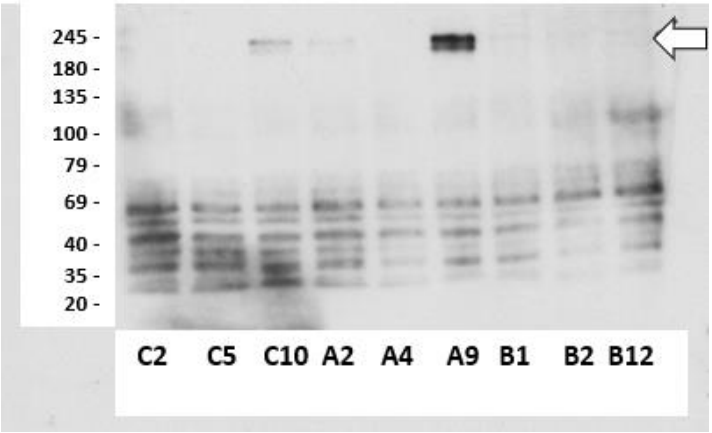

Figure 10. Detection of the dCas9-VP64 joint domain in HEK 293 cells with Western blot. Rabbit dCas9 antibody was used. Expected molecular weight was 231.35 kDa

In terms of visualizing insulin, it needs to be highlighted that since insulin is a relatively small protein (5.7 kDa) it is challenging to detect it using blotting. During the research several blotting attempts have been made to visualize it, but none of them were successful.

3.2. Testing the constructs in H1 stem cells

3.2.1. Activation of insulin transcription in H1 stem cells. Obtaining the CRISPR-edited H1 cells

Once the effectiveness of the designed constructs collectively targeted to switching on the transcription of insulin was validated in HEK 293 cells using qPCR, immunostaining, and also Western blot for certain genes, the research stepped onto the next and most desired phase – stem cells.

For the purpose of the study the commercial research-based embryonic stem cell line H1 was chosen. This H1 cells show a high efficiency in differentiating into various sorts of vital cell types such as cardiomyocytes, pancreatic cells, hematopoietic lineages, demonstrating their versatility in generating various cell types for research and therapeutic purposes [155]. In addition, H1 cells have a number of advantages compared to other stem cell lines like H9 and certain induced pluripotent stem cells (iPSCs). The H1 line has been used in gene editing studies to

enhance safety in stem cell therapies. For instance, the insertion of a suicide gene into the H1 line allows for the selective eradication of undifferentiated cells, reducing the risk of teratoma formation in therapeutic applications. Moreover, the H1 line has been genetically modified to study the effects of specific gene deletions, such as the RB1 gene, while maintaining normal karyotype and pluripotency, which is crucial for understanding genetic diseases and related characteristics [156].

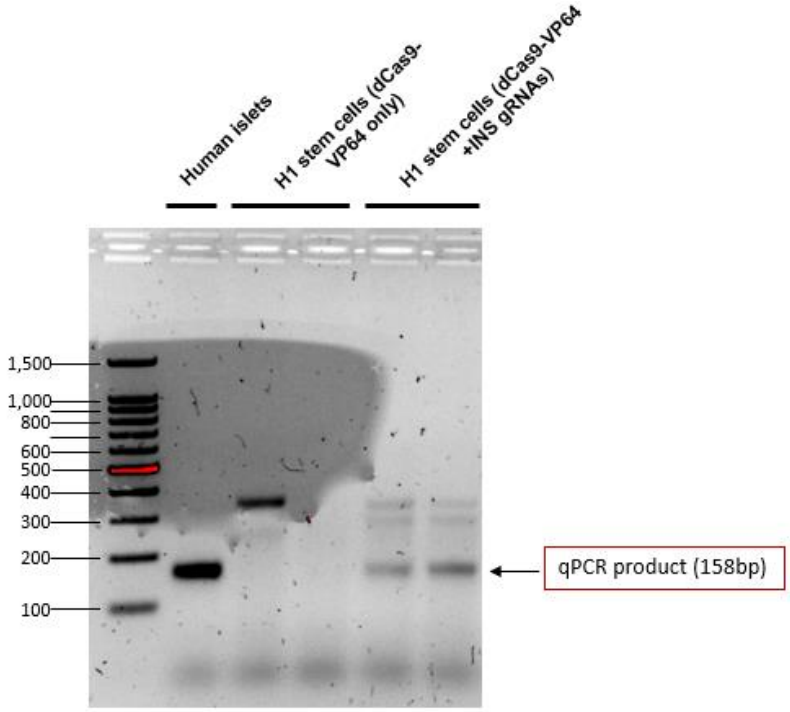

Figure 12. 2% gel electrophoresis of the qPCR samples. The dCas9-VP64 + INS gRNA samples expressed detectable insulin levels compared to negative controls (dCas9-VP64 only). For positive comparison purposes insulin expression levels in human islets were leveraged.

In the course of activating insulin transcription in H1 stem cells, the order of experiments and steps were exactly the same as of the case with HEK 293 cells. The order was the following:

1) transfecting H1 cells with dCas9-VP64 plasmid and obtaining dCas9-VP64 H1 cells stably expressing the genes;

2) transduction of Lenti-INS gRNA into dCas9-VP64 H1 cells to integrate insulin gRNA into the host cells; gRNAs were the same as selected and used for HEK 293 cells.

3) qPCR analysis of insulin gene expression in the cells.

As a result of the experiments, highly detectable (over 900x compared to controls) insulin expression level was observed in the CRISPR-edited (dCas9-VP64 + INS gRNA) H1 embryonic stem cells (Figure 12). The expected qPCR product size was 158 bp which was adequately well shown in gel-electrophoresis. Human islets, used as positive controls, expectedly expressed good level of target product.

As a negative control, H1 cells were used also edited with CRISPR complex but purposefully missing insulin gRNA.

3.2.2. Detection of insulin in CRISPR-edited H1 stem cells with staining technique

As a result of the integration of the CRISPR complex into the H1 cells following the order mentioned above, adequate insulin mRNA levels were detected. Although not required, staining method based on Hoescht dye was used as an additional step to confirm the full expression of the target gene on protein level.

Insulin is a very small molecule in nature (5.7 kDa) and thus Western blotting is not always a suitable technology for detecting such small proteins. Enzyme-linked immunosorbent assay (ELISA) can give favorable results to detect insulin, however, this was not the case in the research as the needed solutions of the assay were not available. Therefore, the protein-detection experiment followed with Hoescht staining. The staining procedure has been fully described above in the Methods section of this work. Generally, the staining lasted two and a half days in duration and as a result insulin was successfully detected in the genome-edited H1 samples (Figure 13) [157].

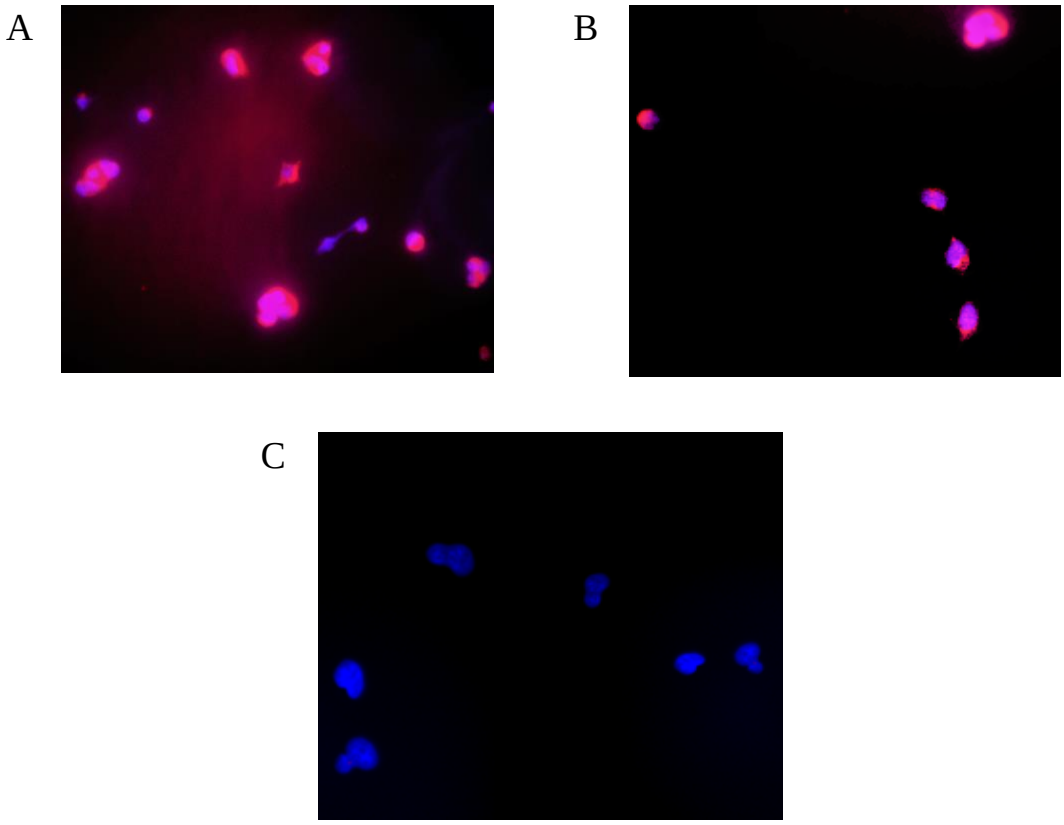

Figure 13. Detecting insulin in CRISPR-edited H1 stem cells with Hoescht dye: A – Positive control: mouse Min6 cells (natural expression of insulin); B – activated insulin expression in H1 stem cells (with CRISPR complex consisting of dCas9-VP64 Lenti INS gRNA); C – negative control (same H1 cells but with no transcription factor added). Viewed using EVOS M5000 40X lenses [157].

3.2.3. Target molecule detection using imaging system

In the course of the research and at various stages respective imaging system was needed to detect and validate expression of target genes in protein level. For this EVOS M5000 imaging system was used which is a versatile tool used in cell culture laboratories for high-throughput imaging applications. The system is equipped with features such as motorized stages and on-stage incubators, allowing for programmable imaging of live cells. This makes it particularly useful for scanning multi-well dishes and creating coherent images through the stitching of multiple adjacent fields (Figure 14).

The EVOS M5000 system is designed to facilitate high-throughput applications and thus enabled the scanning of multi-well dishes and stitching images to produce coherent individual images of each well. This capability was essential for all the cell culture experiments leveraged in the research.

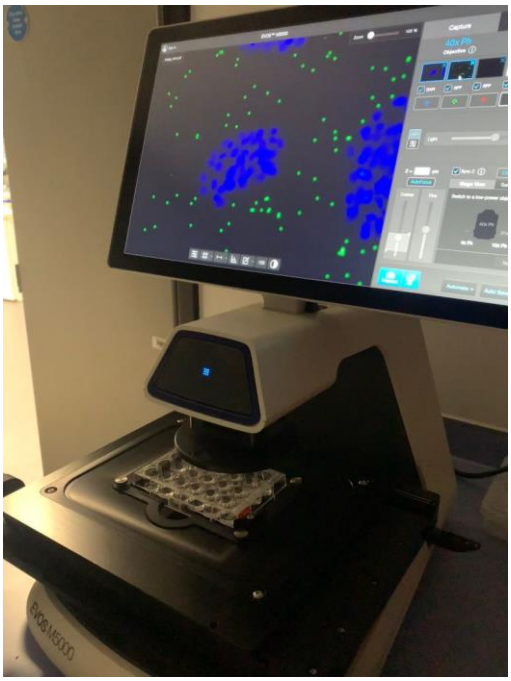

Figure 14. The versatile EVOS M5000 imaging system used for visualization, Thermo Fisher Scientific, USA.

During initial stages of the research, attempts made to quantify images captured by the system encountered several challenges, including high background noise, illumination and stitching artifacts, low contrast, focus inconsistencies, and image distortion. Respectively, these issues negatively impacted the efficiency of image processing and analysis. But later on, by introducing cell counting of tiled images captured by the system, the initial obstacles were overcome, and processing efficiency was improved accordingly.

The use of the imaging system was significant during the differentiation process as each stage contained studying the overall morphological properties of the

cells. The system's EVOS™ 40X lenses were appropriately instrumental and thus were mostly applied for viewing the cell cultures.

3.3. Differentiation of the H1 stem cells to insulin-producing β cells

After successful activation of insulin transcription in genome edited H1 stem cells (Lenti INS gRNA-dCas9-VP64 H1 stem cells) confirmed by qPCR and highlighted by immunostaining method, the next task was to differentiate those cells into pancreatic beta cells. For relevant comparison purposes, ordinary H1 stem cells without any genomic manipulation and CRISPR-edited, but without insulin gRNAs, H1 stem cells were also differentiated to beta cells using the same differentiation protocol described in the Methods section.

As of today, due to acute research need, there are a number of differentiation protocols shared by various research groups. Below are named a few of them which have been discovered in the course of this research. For example, Human Adipose-Derived Stem Cells (ADSCs) - a protocol based on using Tyrphostin9, a PDGFR kinase inhibitor, significantly improved the yield and functionality of β-like cells from ADSCs. The approach enhanced the expression of pancreatic differentiation markers and glucose-stimulated insulin secretion [158]. Next, human amniotic fluid and dental pulp stem cells have been found to differentiate into insulin-producing cells using a multistep protocol. The differentiated cells form islet-like structures secrete insulin in a glucose-dependent manner, offering a non-invasive source for islet regeneration [159]. Furthermore, human pluripotent stem cells were differentiated using a six-stage planar differentiation method which generated pancreatic beta cells with high insulin secretion in response to glucose. This protocol simplifies the process by eliminating the need for 3D culture [160]. Earlier mouse embryonic stem cells-based four-stage protocol minimized neuronal differentiation, enhancing the yield of insulin-producing cells with beta cell characteristics. This method showed potential for improving diabetic conditions in mice [161]. Recently 3D culture systems have been developed. Particularly, a 3D culture system using adipose-derived mesenchymal stem cells accelerated differentiation and improved cell viability and insulin expression, making it suitable for clinical transplantation in the future [162].

In this work, the differentiation process mainly followed the experience of the group of Rezania and the team [163]. However, at certain stages of the protocol slightly modifications and respective adjustments were made in order to obtain the most desired cell line.

3.3.1. Preparation step

Important factor significantly influencing the result of the whole differentiation process is cell seeding. Here confluency of the cells is considered as a vital influencer. Number of repetitions showed us that confluency of 85%-90% is the most suitable considering number of various exposures and environmental challenges the cells are going to be exposed to. The picture below shows the state of cells before the start of the differentiation (Figure 14).

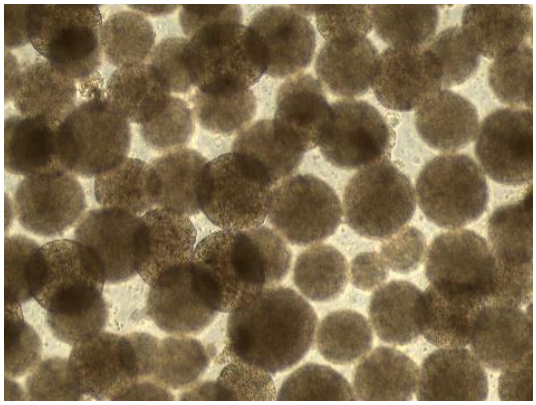

Figure 14. CRISPR-edited H1 stem cells just before the start of differentiation with confluency around 85%-90% after 48 hours of culturing in the E8 medium. Viewed under EVOS™ 20X lenses.

3.3.2. Stage 1: Generation of definitive endoderm cells

Definitive endoderm is a crucial germ layer formed during embryonic development, giving rise to the gastrointestinal and respiratory tracts, as well as organs such as the liver and pancreas. The layer is formed during gastrulation, a process characterized by extensive cell movements. Understanding the formation and differentiation of the definitive endoderm is important for insights into developmental biology and potential therapeutic applications.

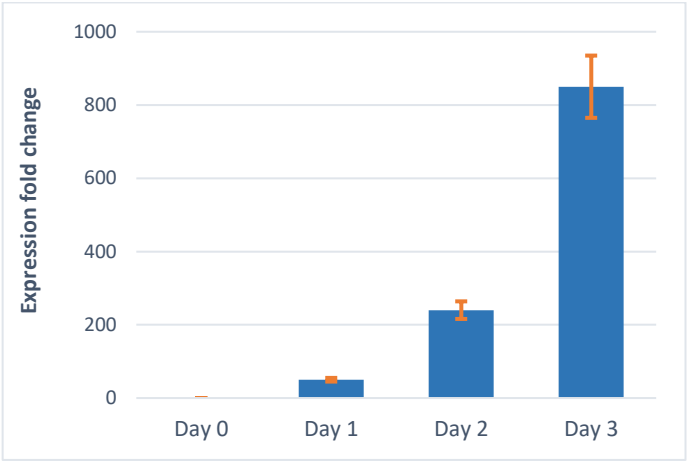

Figure 15. Differentiation stage 1: Expression of FOXA2 key marker by the cells of definitive endoderm stage.

Definitive endoderm cells represent critical component in the development of various organs, including the pancreas and liver. The differentiation of definitive endoderm can be monitored using specific surface markers such as CXCR4, which helps in purifying the definitive endoderm population. Generally, this step is known as a first interim stage in the formation insulin producing beta cells of pancreas

during development of human embryo and its mechanism is well known in science. At this stage first differentiation features start to occur in mother cells and the newly formed interim cells are called definitive endoderm. Reproducing of these events under laboratory conditions is sufficiently studied as well. The differentiation of definitive endoderm involves a dynamic gene expression profile. At this stage the differentiating stem cells express two key marker proteins among other ones: FOXA2 and CXCR4. The essential 8 (E8) medium was used to grow the cells and following the protocol described above, expressions of the marker molecules typical for this stage were studied and analyzed (Figures 15 and 16).

Activin A is commonly used to induce definitive endoderm differentiation from hESCs. It plays a significant role in the early stages of differentiation, and its reduction has been shown to not adversely affect the expression of beta cell markers, thus offering a cost-effective approach to cell production [164]. Additionally, the combination of growth factors such as bFGF, BMP4, and Activin A effectively induce definitive endoderm and pancreatic lineage cells.

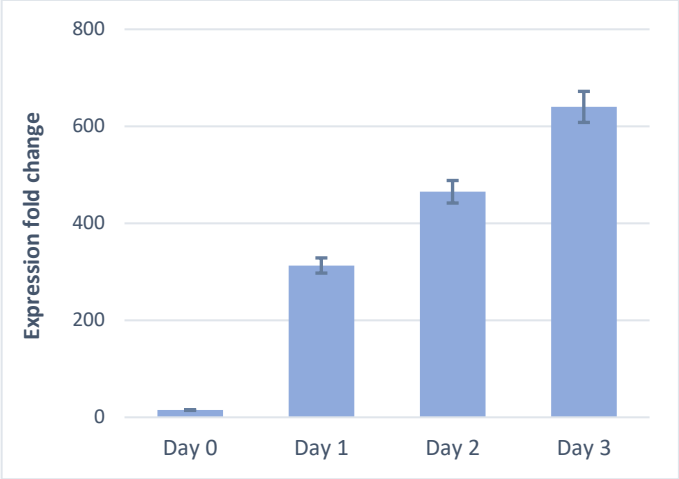

Figure 16. Expression of key CXCR4 marker in cells of definitive endoderm stage.

The duration of the definitive endoderm formation lasted for 3 days. Under the described media conditions, expression dynamics of the FOXA2 was observed as sharp over the duration of 3 days and reached its peak on day 3. The expression was calculated as a fold change and stood at over than 800 times on day3. On the other hand, CXCR4 expression did not change sharply, but rather showed a gradual increase over the days and approximately 650 times fold change increase was reached on the third day. Both expressions' error margins were found to be within 5-10%.

3.3.3. Stage 2: Production of primitive gut tube cells

At this stage the cells were further exposed to the MCDB medium described in the methods section. Meanwhile, the medium was additionally supplemented with ascorbic acid and sodium bicarbonate to maintain the differentiating path of the cells.

The goal at this stage was to keep the expression of FOXA2 (Figure 17), but, in line with other important factors, decrease and completely stop CXCR4's transcription. This is because the expression of CXCR4 is not necessary for the cells of the primitive gut tube taking a further path toward PDX1 transcription.

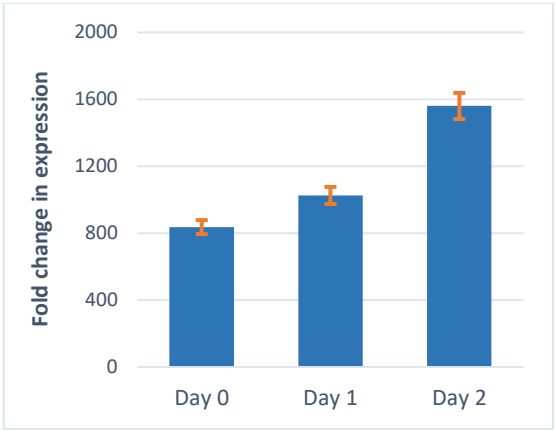

Figure 17. Maintaining FOXA2's expression during formation of the cells of the primitive gut tube

This stage lasted for 2 days and at this point it was important not only keep the expression of FOXA2, but also maintain its further increase as much as possible because it is FOXA2 which plays a crucial role in activating PDX1's transcription in the next stage. Comparing to the definitive endoderm stage, the primitive gut cells expressed FOXA2 in the level of almost 1600 times on day 2 of the stage.

3.3.4. Stage 3: Formation of posterior foregut cells

A main key factor significantly affecting the overall process, and which is upmost to keep supply of is the BLAR medium described above. Particularly, FGF7 and SANT-1 in the medium play key roles in regulating transcription of PDX1 marker and in maintaining its gradual increase over the days of the differentiation. Following the protocol provided, over 25X more fold expression of the PDX1 mRNA was achieved in 2 days compared to day 0 (Figure 18).

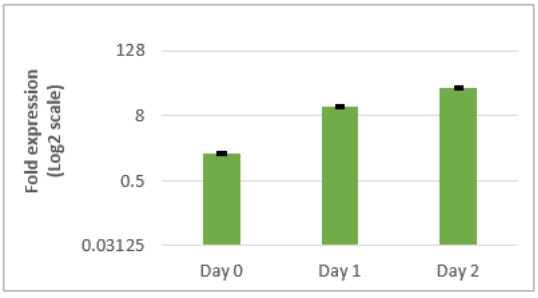

Figure 18. PDX1 expression by the posterior foregut cells (Log2 scale).

It was essential to adequately keep maintaining the incubation of the differentiating cells in the BLAR medium, supplemented with sodium bicarbonate, Glutamax and ascorbic acid.

The importance of this stage is the expression of PDX1 by the differentiating cells. The cells of this step later form numerous internal organs, including pancreas, and many are found in the next differentiation step which is pancreatic endoderm formation.

Morphologically, cells at this stage start assembling into visible clusters that can be visualized under a microscope (Figure 19).

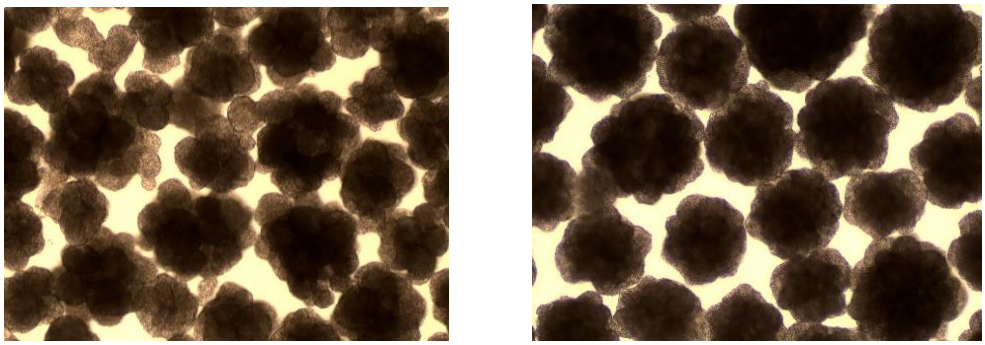

Figure 19. Posterior foregut cells (days 6-7). Viewed under EVOS™ 20X lenses.

3.3.5. Stage 4: Formation of pancreatic endoderm cells

It is at this stage when the main progenitor cells of pancreatic beta cells are formed expressing their key marker NKX 6.1. The NKX 6.1 will remain to be one of the key markers of the cells of each of the following differentiation stage including the last stage when insulin-producing beta-like cells are produced. Therefore, it is essential to keep the PDX1 expressed in this stage as well. Following the protocol provided earlier, almost 60X times for NKX 6.1 and over 70X times increase for PDX1 was achieved at this stage (Figures 20 and 21).

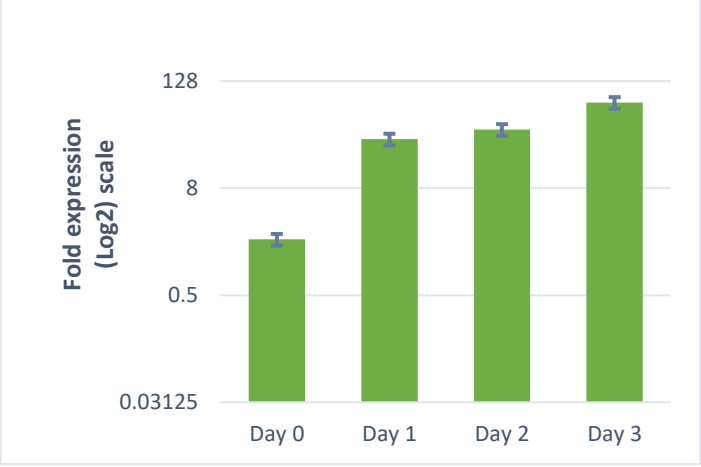

Figure 20. Continued growth in the expression of PDX1 marker by pancreatic endoderm cells.

By the end of this stage, which lasted for 3 days, the cells expressed enough of both the PDX1 and NKX6.1 markers. This was achieved due to BLAR medium and its essential supplements necessary to provide enough resources for the differentiating cells to reach the pancreatic endoderm stage.

NKX6.1 is essential for identifying insulin-producing beta cells and maintaining their identity. It represses alternative endocrine lineage programs, ensuring that beta cells do not convert into other cell types, such as alpha cells, which produce glucagon [165]. NKX6.1 also plays a critical role in regulating glucose-stimulated insulin secretion in beta cells. It suppresses glucagon expression, which is crucial for maintaining the functional state of beta cells. Also, overexpression of NKX6.1 enhances insulin secretion and beta-cell proliferation, mediated through pathways involving Nr4a nuclear receptors and c-Fos6 [166, 167]. Thus, there is a vital role that NKX6.1 regulates in maintaining functional capacity of insulin in the body.

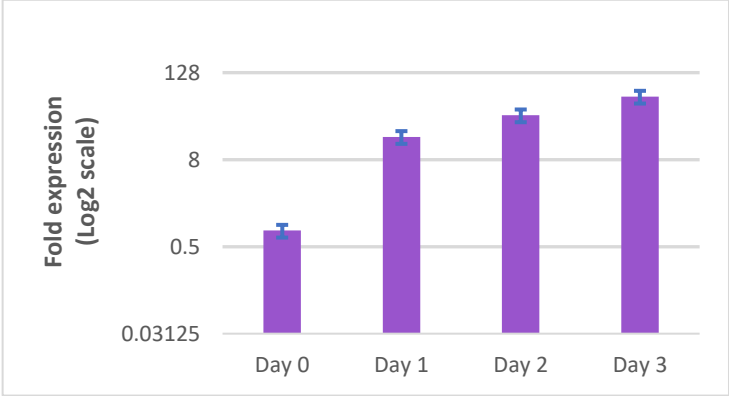

Figure 21. NKX 6.1 expression in pancreatic endoderm cells.

One of the key elements of the BLAR medium used in this stage was 0.25 mM of vitamin C (ascorbic acid). Because it is the ascorbic acid that downregulates NGN3 gene during early differentiation, which is responsible for regulation of many transcriptions including path to polyhormonal cells (insulin, glucagon, somatostatin, and others).

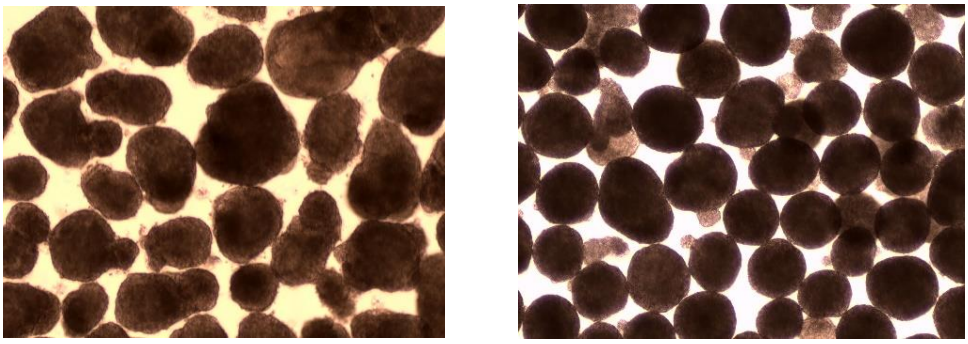

Figure 22. Day 8 pancreatic endoderm cells. Viewed under EVOS™ 20X lenses.

Morphologically, the membranes of the cells at this stage start to form clusters with clear lines and also smooth membranes themselves which allow us to visualize them even with low magnification (Figure 22).

3.3.6. Stage 5: Pancreatic endocrine precursor cells

Key markers the cells are expected to express at the stage of the formation of the pancreatic endocrine precursor cells are PDX1, NKX6.1 and NEUROD1. Meanwhile, a key factor to pay a close and careful attention during this stage was upregulation of NEURODI, a downstream transcription element of NGN3 marker. The NGN3 was inhibited in the stage of endoderm cells' formation, and to enhance its expression it was important to select its highly specific element to be added to the BLAR medium. Indeed, it was not ascorbic acid, and based on results of some experiments and screening, addition of ALK 5 inhibitor to the media provided the highest upregulation of the NGN3. The ALK5 provided over 60X times increase in NEURODI's expression in 3 days while maintaining the continuous rise of PDX1 and NKX 6.1 expressions compared to their levels in the previous pancreatic endoderm formation stage (Figure 23).

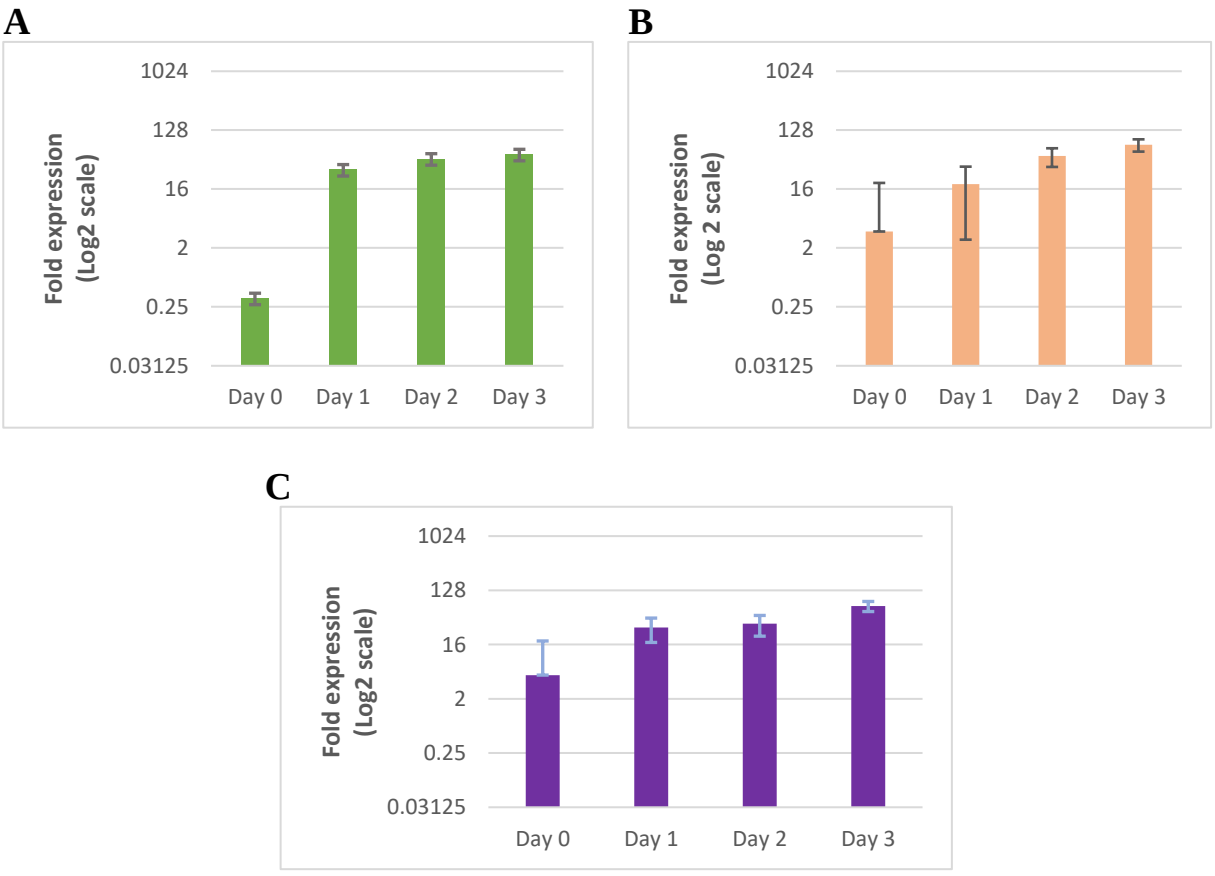

Figure 23. Key markers expressed by pancreatic endocrine precursor cells: A – PDX1; B – NEURODI; C – NKX 6.1. For PDX1 and NKX 6.1 the rises are related to only this stage, though their continuous improvement was maintained over the course of the differentiation.

3.3.7. Stage 6: Formation of NKX 6.1^{+} and insulin^{+} cells

Insulin is first detected in this stage and thus the stage usually lasts from 9 to 15 days. In this research, the stage 6 took 12 days while allowing the cells have an adequate time for regulation of insulin gene transcription and other related transcriptions. In addition, the gamma secretase inhibitor XX was found to be requiring no less than 7 days for fully contributing to the transcription and there should be given additional days for the heparin to help the cells attain more viability. In the end, the cells continued maintaining upregulation of NKX 6.1 and showed acceptable levels of insulin expression with over 20X times more expression compared to controls (Figures 24 and 25).

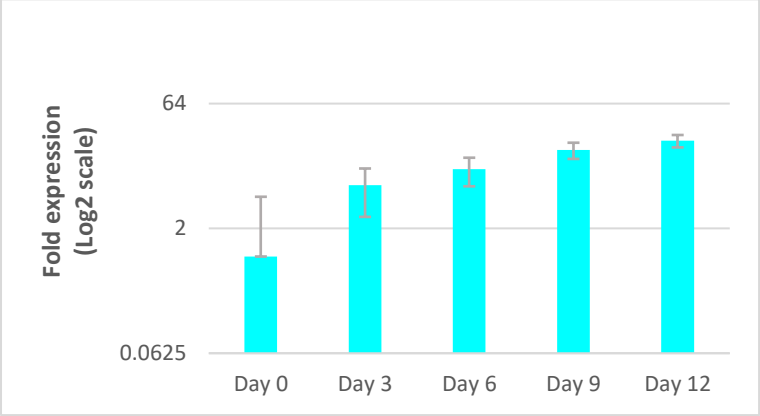

Figure 24. Expression of insulin by the beta cells derived from CRISPR-edited H1 stem cells.

Another key element of the medium at this stage was T3, which was identified as a molecule directly regulating expression of insulin and a few mature beta cell markers. In addition, T3 worked out best in conjunction with ALK inhibitors.

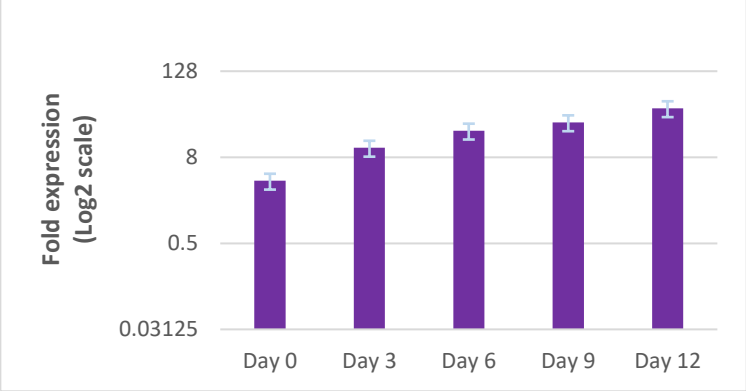

Figure 25. Expression of NKX 6.1 marker by the obtained beta cells.

The characteristics of NKX6.1$^+$ and insulin$^+$ cells is crucial for understanding the development and function of the differentiated pancreatic beta cells, which are responsible for insulin production. Research results obtained at this level of differentiation is particularly relevant for developing respective therapies and clinical applications.

Morphologically, in this stage the NKX 6.1$^+$ and insulin$^+$ cells almost finished forming clear clusters of cell groups (Figure 26). Also, it was quite clear to visualize some cells which had not formed clusters, and these cells were assumed to undergo apoptosis some time soon due to growing and continuing effect by the glucose in the medium.

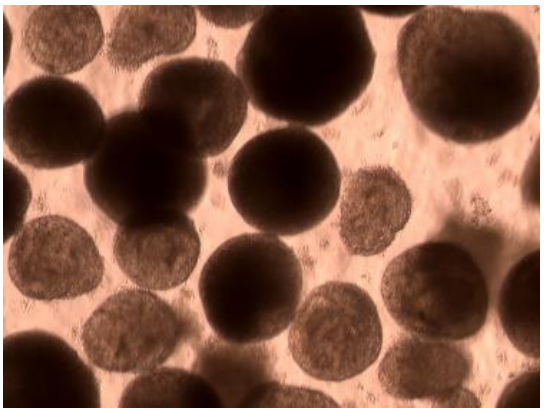

Figure 26. Microscope image of NKX 6.1$^+$ and insulin$^+$ cells. Viewed under EVOS™ 20X lenses.

Although the expression of insulin took place by the differentiated cells at this stage, the expression level of the hormone was far below compared to what natural human islets demonstrate in a regular time. In addition, the cells did not yet express MAFA, another distinguishing marker typical for mature beta cells as pancreatic human islets.

3.3.8. Stage 7: Generation of NKX 6.1$^+$, insulin$^+$ and MAFA$^+$ cells

As mentioned above, MAFA is an important feature that naturally insulin-producing cells have. The marker is responsible for regulation of insulin gene transcription as well as for inducing its activation in response to glucose stimulation. In nature, human islets have elevated level of MAFA. Thus, one of the main goals of the differentiation process was to reach at least 50% of the natural level of MAFA expression at the end of the differentiation.

In order to do so, a number of chemical compounds were screened by research teams for the ability of compounds induce MAFA expression while not affecting desired growth of insulin and NKX 6.1 expression.

Ultimately, among others, gamma secretase inhibitor XX has been identified as the best compound for this purpose. Using the inhibitor XX provided almost 80X more insulin expression, around 150X – for NKX 6.1 and more than 30X - for MAFA marker compared to the previous stage (Figure 27).

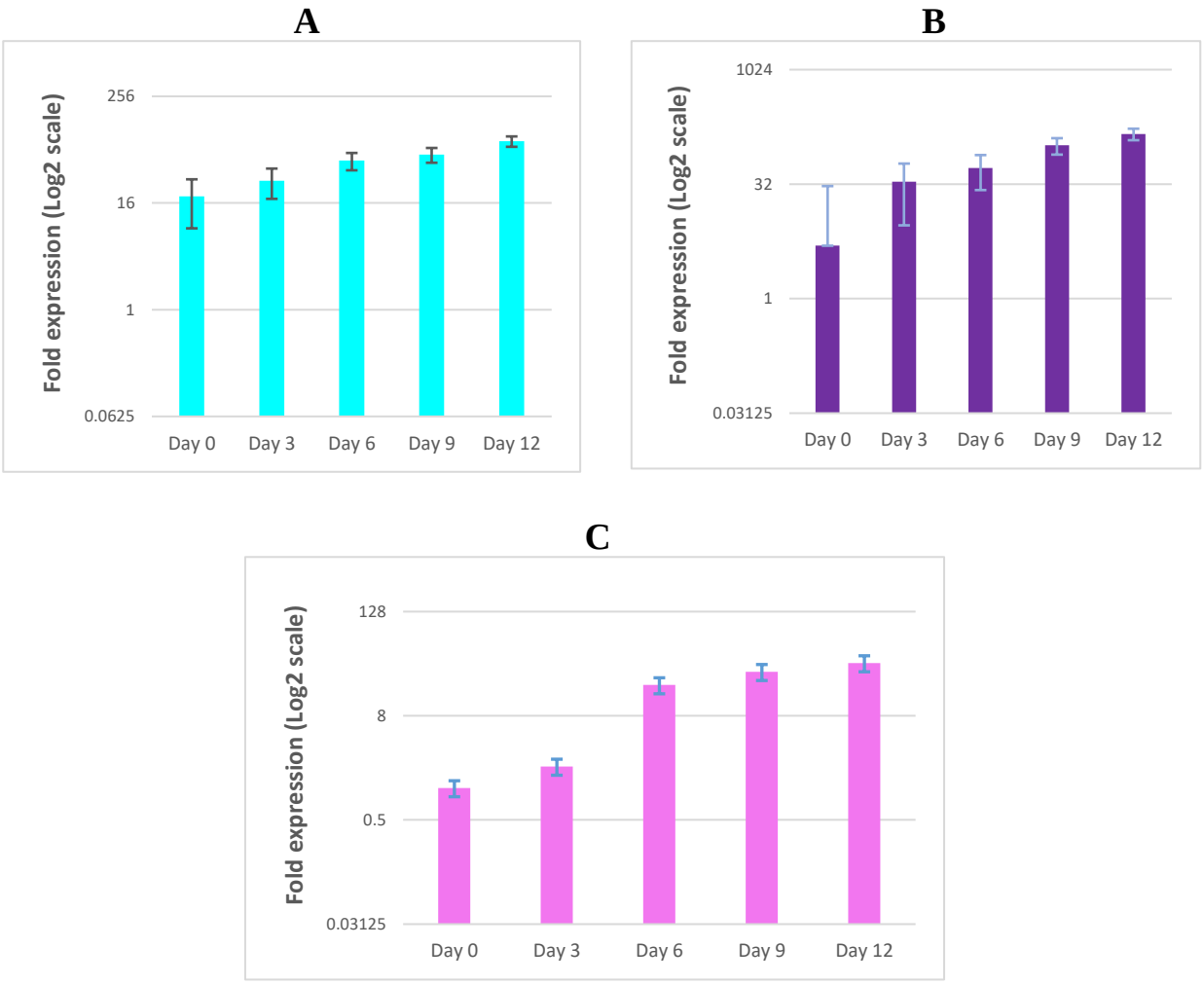

Figure 27. Expression of insulin (A), NKX 6.1 (B) and MAFA (C) by the differentiated beta cells.

MAFA is a potent activator of insulin transcription, specifically in beta cells. It binds to the insulin promoter and enhances insulin mRNA production, which is crucial for insulin synthesis and secretion. MAFA is essential for the differentiation of beta cells during development and their functional maturation. It is involved in the second phase of beta cell differentiation and is absent in cells lacking this phase, indicating its role in beta cell development [168]. MAFA regulates glucose-stimulated insulin secretion by influencing the expression of genes involved in glucose metabolism and insulin secretion pathways, such as glucokinase and GLUT2. MAFA is also crucial for maintaining the mature beta cell phenotype. Loss of MAFA can lead to dedifferentiation of beta cells, reducing their ability to produce insulin and potentially converting them into other cell types, such as alpha cells [169].

Formation of NKX 6.1$^+$, insulin$^+$ and MAFA$^+$ cells from NKX 6.1$^+$ and insulin$^+$ cells lasted for 12 days. Moreover, it was essential to change the culture's medium every day.

Thereafter, the cells were analyzed for expression of somatostatin and glucagon. During the previous stage when the cells first started to express insulin, they were also expressing the other two hormones in levels no lower than of insulin. Thus, while achieving the expression of MAFA, it was also important to decrease the transcriptions of those hormones. After a few modifications of the protocol, finally relative downregulation of the target genes was achieved (Figure 28).

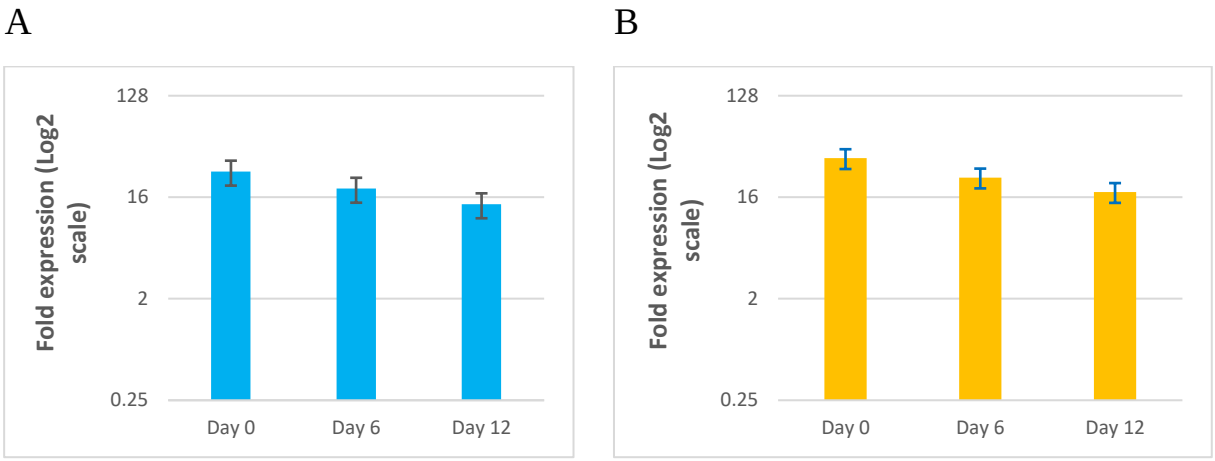

Figure 28. Decreased expression of glucagon (A) and somatostatin (B) by the differentiated beta cells during the days of the last stage of the differentiation.

Comparably, the decrease of glucagon was achieved slightly faster and easier than of somatostatin. This is probably because in nature glucagon is produced by pure alfa cells of pancreas while somatostatin's release is more complex, and also that insulin is included in its regulation.

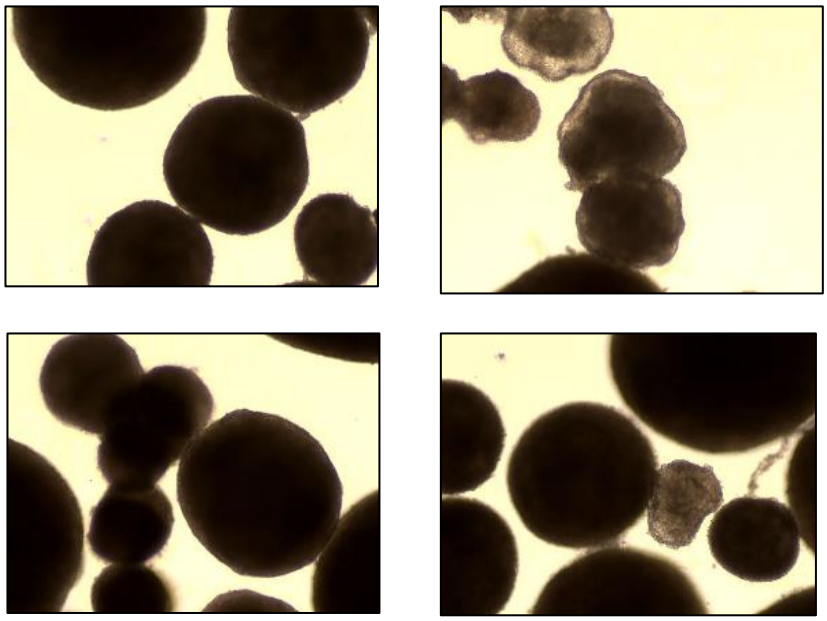

Figure 29. Microscope view of the differentiated insulin-producing beta cells. Viewed under EVOS™ 10X lenses.

In addition, majority of the differentiated cells had clear margin membranes and only a few of them still were in the form of clusters (Figure 29), although natural human islets are purely clusters of cells. Addition of cluster forming agents to the media might induce intercellular communication and ultimately help them form the islets, however this was not performed since any change in the media might have significantly affected the desired transcriptions.

Therefore, at this point the differentiation process of the CRISPR-edited H1 human embryonic stem cells toward insulin producing pancreatic beta cells was completed. The process consisted of 7 stages with overall duration of 38 days of distinct cell culture experiments.

3.4. Analysis of insulin production in the differentiated pancreatic β-cells

After the differentiation and the related tests were over, the next goal was to check the level of insulin production by the differentiated cells. Real time qPCR was leveraged for this purpose.

As was mentioned above, among CRISPR-edited H1 cells we had two groups: one with full CRISPR complex and another without insulin gRNA as a negative test samples. Therefore, when it came to test insulin synthesis levels, both of these two groups were used and compared against each other. Additionally, these groups underwent the same path during the differentiation and also experienced exactly the same treatment with CRISPR complex editing. Meanwhile, as a positive control samples, human islets were utilized as they demonstrate naturally adequate level of the hormone production.

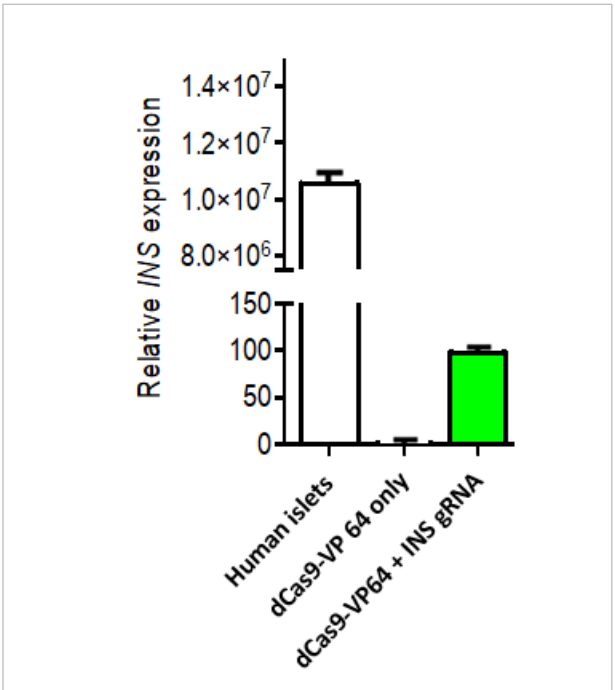

Figure 30. Comparison of insulin expression in natural human islets (first column), pancreatic beta cells derived from CRISPR-edited (but without insulin gRNA) H1 stem cells (middle) – negative control and beta cells derived from CRISPR-edited (with insulin gRNA) H1 stem cells – test samples.

As qPCR showed, CRISPR-edited H1-derived beta cells had roughly 100x more insulin production level as compared to their peers but derived from another group of H1 cells treated with CRISPR complex lacking insulin gRNA (Figure 30). This confirms the vital role which gRNA plays for effective and targeted gene expression regulation using synthetic transcription factor-based CRISPR construct. Compared to natural human islets, the test samples expectedly demonstrated much lower levels of the hormone.

The next part of the analysis was about comparing a difference (if any) in insulin expression between the pancreatic beta cells derived from ordinary (not genome-edited) H1 cells and CRISPR-edited test samples. Interestingly, a significant difference was detected by qPCR in the levels of the insulin mRNA between the groups (Figure 31).

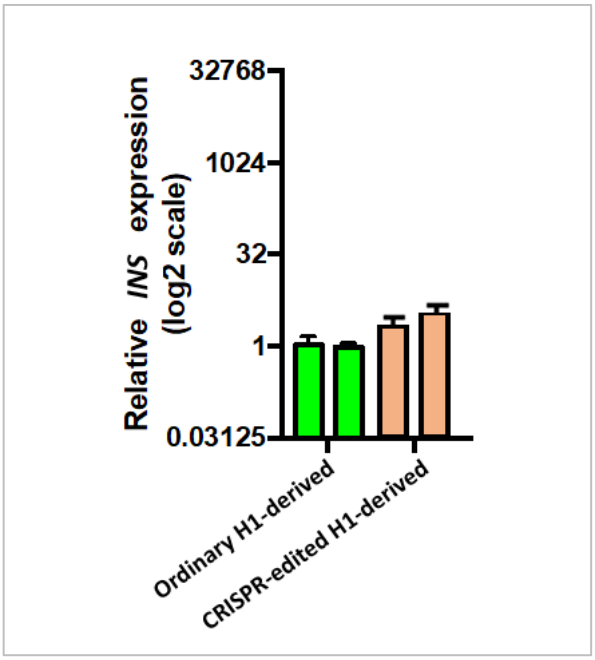

Figure 31. Different insulin production levels by beta cells derived from ordinary (non-CRISPR edited) H1 stem cells and the beta cells derived from CRISPR-edited H1 stem cells.

3.5. Assessing glucose-responsivity feature of the obtained insulin-producing cells

Producing insulin in response to changes of glucose concentration in the media is a main and distinguishing feature of pancreatic beta cells. In nature, human beta cells sense the change of glucose amount in the blood and quickly and perfectly response to it by secreting the hormone. Therefore, the ultimate goal of the all research being carried out on this topic is to obtain healthy, functioning beta cells that would carefully perform their job. This feature of the cells needs be assessed in the course of the study.

In this project, to perform this experiment all the cells including the controls were cultured in 12-well plate for 2 consecutive days. There were 4 groups of cells:

negative control, natural H1, CRISPR-edited H1 and human islets as positive control. Each group of cells were seeded in 3 wells. After 1 day of cultivation, the confluency in the wells reached 75%. On day 2 of the cultivation, all the cells were incubated for 30 minutes at 5 mM glucose for equilibration purposes. Then 5mM, 15mM and 25mM of glucose was added to each well (each group receiving each concentration) and the cells were kept incubated for 120 minutes in total. After the time past, the media was collected for analysis which demonstrated quite interesting and at the same time very promising results (Figure 32).

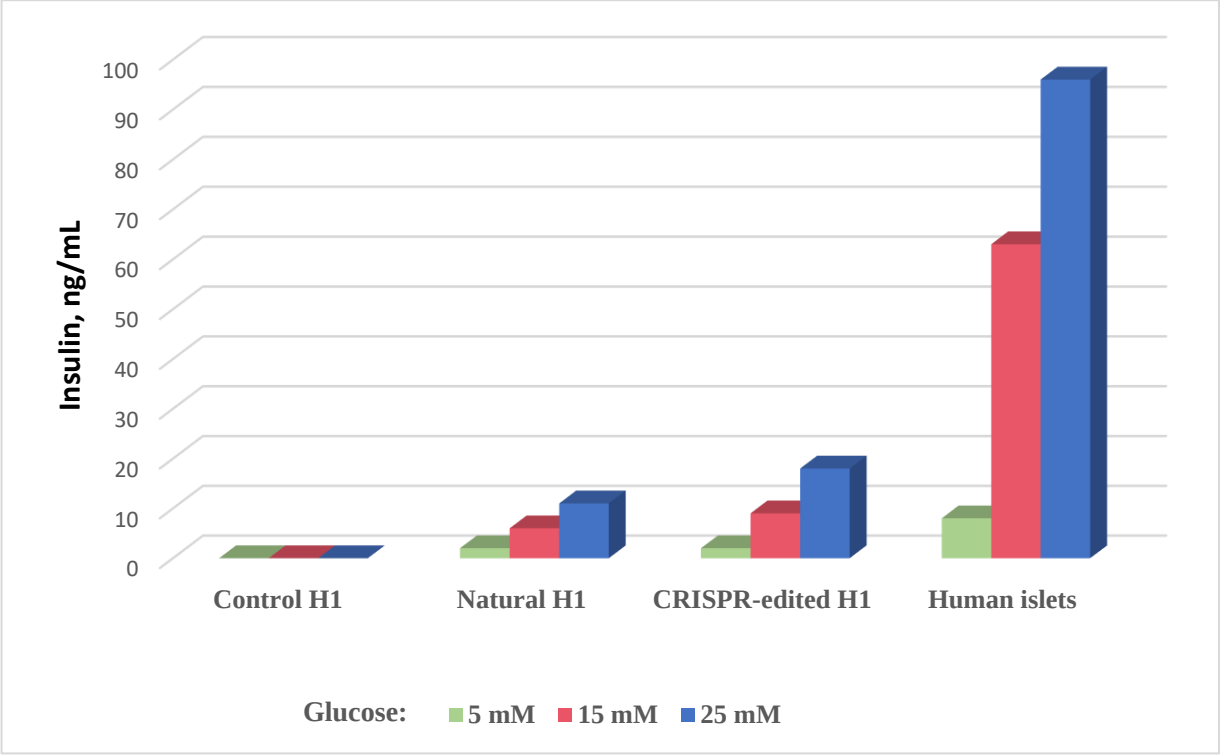

Figure 32. Comparative assessment of glucose-responsivity of the obtained differentiated cells in regard to other controls: Control H1 – CRISPR-edited H1 cells but without insulin gRNA (no expression, negative control); Natural H1 – ordinary cells; CRISPR-edited H1 – research cells; Human islets – native insulin-producing pancreatic cells (positive control).

When the cells were incubated at each concentration of glucose, the wells of the plate contained 1 ml of media. Then 1 ml glucose was added to each well in a way that the final concentration of the glucose in the media and in each well would be as needed: 5 mM, 15mM and 25 mM.

3.5. Summary of the results

In this work, a new approach of cellular therapy for diabetes type I has been developed by producing insulin-producing pancreatic beta cells. CRISPR-Cas9 technology based on synthetic transcription factors and equipped with target-specific gRNA showed adequate functionality in obtaining beta cells with enhanced and

decreased insulin transcription. For this to happen, several guide RNAs (gRNAs) were specifically designed as complementary to insulin's promotor region. Specific primer designing tool GuideMaker was used for creating gRNAs. Then the most effective ones were selected using nuclease Surveyor assay by specific cutting ability of the target sequence. By integrating with deactivated Cas9 nuclease (dCas9), which is a part of CRISPR complex, the gRNAs direct the complex to the promotor of the target gene. Based on available data shared by other research groups, the most effective 2 gRNAs were sorted out and packaged into lentiviral vector where each gRNA was driven by a separate promotor. Once transformed to host cell, gRNA interact with dCas9 in a specific manner and form a firm connection. This interaction leads to transcription activation machinery start the process.

Synthetic transcription activation factor VP64, fused with the dCas9 domain, will induce insulin transcription upon entering host cell together with the dCas9. Interaction between gRNA and dCas9, brings VP64 to close enough proximity to the promotor of the target gene. This mechanism of combination functioned well and was confirmed by successful activation of insulin transcription in ordinary HEK 293 cells. Next, the goal was to check feasibility of further modulating the activated insulin expression. For this purpose, endogenous transcription repressor KRAB protein was chosen and integrated into the host HEK cells as a part of dCas9-KRAB plasmid. The modification also worked well demonstrated by significant reduction in the expression of insulin mRNA in the HEK 293 cells. This part was done for two reasons where apart from testing feasibility of the proposed gene regulation complex it was found that it might have been necessary to have lowered degree of insulin production during different stages of disease progression in human subjects.

Role and mechanism of action of transcription activator and repressor domains, VP64 and KRAB, respectively, are well studied in science. Any endogenous gene's transcription in human body is regulated with many factors collectively called as transcription factors. They are mainly native molecules. Additionally, for research purposes there are transcription factor molecules created specifically by scientists and their action is widely used in many research studies. As results described above, the transfection of target transcription factors into HEK 293 cells were completed and their expression was achieved which was confirmed with qPCR. The success of the combined efforts of the VP64 with gRNAs were enhanced insulin mRNA in the HEK 293 cells.

Thus, when all the genetic modification processes were completely tested in the HEK293 cells and the CRISPR-based insulin gene regulation machinery's effectiveness was proved, the research continued to the next stage. At this point all the previously described procedures were repeated and tested in H1 line of embryonic stem cells. The procedures were completed with confirming significantly enhanced insulin transcription in the H1 cells and was followed by in vitro differentiation of the CRISPR-edited stem cells toward pancreatic beta cells. Additionally, for comparison purposes two other groups of H1 cells were leveraged: ordinary (not genome-edited) ones and ones with CRISPR-edited genome but without gRNA part.

Before proceeding to the differentiation, Hoechst dye-based immunostaining was performed on the genome-edited H1 cells for confirming the expression of the target gene by detecting insulin as protein. Mouse native Min6 cell line has been chosen as a control for comparison with the H1 cells with increased expression of insulin. Hoechst staining is based on antibody specificity and thus detects only those cells producing insulin. It should also be noted here that to date there are only a few scientific literatures available describing studies devoted to immunocytochemically detecting insulin. As a result of the staining, activated transcription of insulin gene and consequently insulin hormone was successfully detected, and the level of expression (color) was statistically significant.

The next step was to perform the differentiation process. As mentioned above, to date several differentiation protocols are available describing production of insulin producing beta cells from stem cells, both embryonic and induced pluripotent. Considering that formation of beta cells during embryonic development in nature is well studied and relying on available literature, the differentiation process was completed and desired transcriptions of key markers in all steps were achieved.

To date, almost all publicly available research studies have described successful production of pancreatic endoderm cells, in other words cells stably expressing PDX1 marker. Important stages occur after this step when cells are expected to express NKX 6.1 marker which is a key property of cells making a journey toward becoming insulin producing cells. In this work, insulin producing beta-like cells were successfully formed and the challenge was to lower the expression of glucagon with somatostatin and at the same time to achieve expression of MAFA marker. The challenge was adequately overcome and differentiated cells produced MAFA and less glucagon and somatostatin compared to human islets. Moreover, the cells maintained an increase in the expression of insulin hormone.

Once the differentiation was completed, analysis was performed on studying level of insulin expression in the obtained beta cells derived from CRISPR-edited H1 cells. Compared to the negative control group, which the same H1 cells but only without integration of gRNAs, the fully edited and differentiated cells expressed a lot more level of insulin production. However, their insulin level was a lot less compared to insulin synthesis in natural human islets.

It was also experimentally shown that β-cells obtained from the CRISPR-edited H1 stem cells demonstrate more insulin expression compared to β-cells obtained from non-transfected ordinary H1 cells. This shows that the use of CRISPR technology in the modulation of endogenous insulin in stem cells can still have a positive effect on the expression of the hormone even in the differentiated β-cells.

Therefore, the study showed the possibility of regulating insulin transcription by synthetic-transcription factors-based CRISPR-Cas9 technology first in human HEK 293 cells, then in the H1 embryonic stem cells. It has also been shown that the genetically modified H1 cells can be differentiated to pancreatic β-cells and without losing expression of key genes of the CRISPR complex.

CONCLUSION

Diabetes remains one of the biggest healthcare burdens of the century with over five hundred thousand registered patients over the world. Type I diabetes is a health condition where absolute deficiency of insulin has taken place mainly due to autoimmune-driven destruction of pancreatic β-cells. Research also shows that genetic factors might play a significant role in the development of the disorder. According to statistics, type I diabetes accounts for around 10% of all the diabetic cases.

According to the latest data from the International Diabetes Federation, there were more than 800,000 diabetic patients in Kazakhstan in 2021. Experts have calculated that during the period of 2014-2019, the mortality increased 4 times for type 1 diabetes, and 6 times for type 2 diabetes in the nation. In addition, according to another source, during the period from 2014 to 2021, the periodic prevalence rate of type 1 diabetes in minor patients in the republic increased from 48.8 to 179.1, whereas the mortality rate increased from 0.18 to 0.67.

The only available therapy today for diabetes type I is insulin injection where the patient continuously receives a dosage of the hormone throughout a day. One and the leading potential cure is transplantation therapy, which is devoted to restoring the deficiency of the hormone insulin. However, worldwide scarcity of donor material (organ, tissue, cells, etc.) and the one with suitable features, preserved function of the cardiovascular system and confirmed brain death, makes the therapy not available for significant amount of the patients. In this regard, scientists have been rigorously working to find a solution and therefore embryonic stem cells are considered as a potential option since they can give rise to any type of cells in human body including the insulin producing cells of pancreas.

Due to lack of effective therapy, today there is a high need to develop new effective strategies to cure the disease, including the problem of hyperglycemia and hypoglycemia in patients. The only possible and promising therapy today is to create a new source of β-cells that can perform two critical functions upon transplantation: proper maintaining of blood glucose levels and glucose-dependent insulin secretion (sensitivity to changes in blood concentrations). Thus, as a suitable solution is to use embryonic stem cells that can give a rise to any cell type in the human body. Applying genome editing approach as a technology for efficient and targeted editing of eukaryotic genomes to produce insulin-producing cells with tunable insulin regulation derived from the stem cells could offer viable treatment option. Indeed, over the past decade, the field of genetic editing has experienced rapid development with the CRISPR/Cas9 method being one of the revolutionary discoveries.

In this work, development of a targeted and CRISPR-based approach for modulating insulin transcription and thereby obtaining insulin-synthesizing cells derived from genetically modified embryonic stem cells as a new potential approach for the treatment of type 1 diabetes was described.

Designs of several guide RNAs (gRNAs) have been created to direct the CRISPR complex to the promoter of the insulin gene, of which 2 most effective ones were selected. A new vector has been created based on lentivirus to deliver selected

gRNAs to target cells. Plasmids with inactive nuclease dCas9 and transcription activator VP64 and transcription repressor KRAB, dCas9-VP64 and dCas9-KRAB, respectively, were obtained and validated. A stable HEK 293 cell line expressing proteins of the plasmids was obtained.

The effectiveness of the developed CRISPR complex (dCas9 nuclease, gRNA, transcription regulator) in HEK 293 cells was tested by transducing the previously obtained dCas9-VP64 and dCas9-KRAB HEK 293 cells with the lenti insulin gRNA vector. As a result, it was shown that the developed genetic approach is able to activate and further reduce the expression of insulin in the ordinary HEK 293 cells.

The developed constructs, in particular the dCas9-VP64 plasmid and the lentiviral vector with insulin gRNA, have been introduced into H1 stem cells. As a result, as validated earlier, the CRISPR system significantly enhanced insulin transcription in the H1 cells.

In vitro directed differentiation of the genetically modified H1 stem cells into insulin-synthesizing pancreatic β-cells was performed using the most suitable protocol at the time of the research. As results of the differentiation, the final cell line expressing key markers of natural β-cells such as NKX 6.1, MAFA and insulin was obtained. Statistically significant expressions of each gene were confirmed. The resulting cells had respectively higher level of insulin expressed compared to the expression of somatostatin and glucagon hormones.

The obtained cells expressed higher insulin production in comparison to β-cells derived from non-genome edited H1 cells indicating positive effect of the CRISPR editing to the synthesis of the final product. Meanwhile, the level of insulin expression in the CRISPR-edited H1-derived β-cells were expectedly lower than in human islets.

In conclusion, the following statements can be drawn based on the results obtained in the work:

1. Insulin gRNAs can be designed and respectively packaged into a lentivirus-based vector which effectively delivers them into host cells. The final vector contained a reporter gene dsRED and two guide RNAs targeted to the promoter of the gene of insulin;

2. Synthetic transcription factors are feasible be linked with deactivated Cas9 nuclease as a part of relevant plasmid. The linkage provides the regulator interact with the target sequence once dCas9 interacts with the gRNA. Upon introduction in host cells, the transcription activation factor VP64 and repression domain KRAB enhances and downregulates insulin transcription, respectively;

3. CRISPR gene regulation complex consisting of the CRISPR-dCas9-VP64 plasmid and the lentivector with INS gRNA is effective in activating insulin expression in human HEK 293 cells. Addition of dCas9-KRAB plasmid to the cells causes competition between VP64 and KRAB for interacting with insulin gRNAs and thus reduces insulin expression level;

4. The obtained CRISPR system for insulin expression regulation demonstrates similar level of effectiveness upon introduction to H1 embryonic stem

cells. Meanwhile, those H1 cells missing insulin gRNA respectively demonstrate no level of the expression of the target product;

5. Directed *in vitro* differentiation of the CRISPR-edited H1 stem cells to pancreatic β-cells provides an approach for obtaining new line of insulin-producing cells. The obtained cells are capable of expressing more insulin than ordinary (non-genome-edited) H1-derived β-cells, but expectedly much less insulin compared to natural islet cells;

6. It has been determined that the obtained CRISPR-edited H1-derived insulin producing cells are a way less sensitive to changes in glucose concentration in the media compared to natural islet cells, but slightly more sensitive than ordinary H1-derived β-cells.

Results of the research led to obtaining a new scientific knowledge on diabetes in terms of the applicability and effectiveness of using CRISPR-based genome editing technology. An approach of obtaining insulin-producing cells from genome-edited stem cells for potential transplantation was assessed. The obtained results have potential to mark the beginning of the next stage of the experiments – enhancing the functionality of the differentiated cells in terms of better glucose-responsive property and more effective insulin regulation.

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