ABSTRACT

of the dissertation for the degree of Philosophy Doctor (Ph.D.) in the specialty 6D060700 - "Biology"

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

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 externally to maintain their life. Otherwise, blood sugar levels will keep rising, leading to a number of unfavorable consequences, including death. Therefore, the most potential treatment for type 1 diabetes is transplantation: either healthy insulin producing β-cells, the islets (Langerhans) or the entire pancreas. In any ways, acute shortage of donor material greatly limits the opportunities. In this regard, use of cell technologies built on stem cells (SC) as a primary source of any tissue in the body is considered as the most potential solution. And on top of this, capacity of recently discovered CRISPR technology, a genome editing method, obtaining new cell lines with improved qualities has become real and could be feasible in diabetes treatment.

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

The following tasks are 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 plasmids where dCas9 is respectively linked with a

synthetic transcription activator (VP64) and a repressor (KRAB) for regulating 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, 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 ultimately.

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 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 effectiveness in H1 embryonic stem cells similar to that of 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.

The research described in this dissertation was performed at the M.A.Aitkhozhin Institute of Molecular Biology and Biochemistry (Almaty, Kazakhstan). The research in parts was 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.

A part of the research was completed at the Diabetes Research Center at the University of British Columbia (Vancouver, Canada) within grant funding of Juvenile Diabetes Research Foundation (now – “Breakthrough T1D”).

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 instructions (methods, protocols). Most of the methods mentioned in this research have been developed by scientific community

previously, some methods such as differentiation of stem cells to insulin producing pancreatic cells as well as immunostaining protocols belong to the Diabetes Research Group of the University of British Columbia. The PhD-candidate by himself has performed all the analysis of the results obtained, drawing figures, searching for literatures and in the end writing of this dissertation.

Research approbation. The results of the research have been presented 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 international scientific conferences.

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