UNIVERSITY OF CRETE

MEDICAL SCHOOL

Master thesis: "Employing CRISPR/Cas9 to study the *GLUD*1 and/or *GLUD*2 knock out effect on HEK293 cell cultures."

Μεταπτυχιακή εργασία: « Χρήση του CRISPR/Cas9 για τη μελέτη της επίδρασης που προκαλεί η απαλοιφή του *GLUD1* ή/και του *GLUD2* γονιδίου σε HEK293 κυτταροκαλλιέργειες.»

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Περίληψη

Θεωρητικό υπόβαθρο: Η γλουταμική αφυδρογονάση (GDH) είναι ένα μιτοχονδριακό ένζυμο που βρίσκεται σε αφθονία και καταλύει την αντιστρέψιμη αλληλομετατροπή του γλουταμικού σε α-κετογλουταρικό και αμμωνία χρησιμοποιώντας το NADP(H) και το NAD(H) σαν συμπαράγοντες. Στα θηλαστικά, η GDH πιστεύεται ότι ευνοεί την παραγωγή του α-κετογλουταρικού το οποίο στη συνέχεια χρησιμοποιείται για παραγωγή ενέργειας μέσω της εισόδου του στον κύκλο του Krebs. Στους ανθρώπους, η GDH κωδικοποιείται από δύο γονίδια, το *GLUD1* και το *GLUD2*, τα οποία έχουν μεγάλη ομολογία.

Στόχος της μελέτης: Ο στόχος αυτής της μελέτης ήταν να εξεταστεί η επίδραση που έχει η απαλοιφή της *GLUD1* ή της *GLUD2* (ή και τον δύο), που πραγματοποιήθηκε σε ανθρώπινες κυτταρικές σειρές, στην κυτταρική βιωσιμότητα και τον μεταβολισμό.

Μέθοδοι: Στην παρούσα μελέτη, χρησιμοποιήσαμε το CRISPR/Cas9 σύστημα (ένα νέο εξαιρετικά αποδοτικό σύστημα που χρησιμοποιείται ολοένα και περισσότερο για την εξάλειψη γονιδίων) προκειμένου να απαλείψουμε είτε και τα δύο, είτε ένα εκ των GLUD1 και GLUD2 γονιδίων κάθε φορά, σε HEK293 κυτταροκαλλιέργειες. Για το λόγο αυτό, σχεδιάστηκαν δύο ζεύγη συμπληρωματικών μονόκλωνων RNA (sgRNAs) ειδικά για την hGDH1 και δύο ζεύγη ειδικά για την hGDH2. Στη συνέχεια, κάθε sgRNA κλωνοποιήθηκε στον pX458 πλασμιδιακό φορέα ο οποίος περιέχει το γονίδιο Cas9.

Αποτελέσματα και συζήτηση: Λόγω του περιορισμένου χρόνου, δεν καταφέραμε να φτάσουμε στο στάδιο της έκφρασης αυτών των πλασμιδίων σε HEK293 κύτταρα (η διαδικασία αυτή πραγματοποιείται αυτό τον καιρό). Ο τελικός στόχος είναι η μελέτη της κυτταρικής βιωσιμότητας και του μεταβολικού προφίλ HEK293 κυττάρων από τα οποία έχουν προηγουμένως εξαλειφθεί οι hGDHs.

Abstract

Background: Glutamate dehydrogenase (GDH) is an abundant mitochondrial enzyme that catalyzes the reversible interconversion of glutamate into a-ketoglutarate and ammonia, using NADP(H) and NAD(H) as cofactors. In mammals, GDH is believed to favor the production of a-ketoglutarate which in turn is used for energy production through its entry to the Krebs cycle. In humans, GDH is encoded by two different genes, *GLUD1* and *GLUD2*, that are highly homologous.

Aim of the study: Aim of this study was to examine the effect on cell viability and metabolism of knocking out either *GLUD1* or *GLUD2* (or both) in human cell cultures.

Methods: In the current study we employed the CRISPR/Cas9 system (a new highly efficient system of gene elimination that is increasingly used in molecular biology) to delete either both or each one of the*GLUD1* and *GLUD2*genes at a time, in HEK293 cell cultures. For this reason, two pairs of complementary single guide RNAs (sgRNAs) specific for hGDH1 and two pairs specific for hGDH2 were designed. Then, each sgRNA was cloned into pX458 plasmid vector that contains the Cas9 gene.

Results and discussion: Due to limited time, we did not reach the stage of expressing these plasmids in HEK293 cells, (this is currently pursued).The final aim is to study cell viability and metabolic profile, after eliminating hGDHs from these HEK293 cells.

1. Introduction

1.1. Brain energy metabolism

Even though brain constitutes about 2% of the total body weight, it requires an amount of energy equivalent to 25% of total body glucose usage. Depending on tissue specificities, glucose can be processed through different metabolic pathways. In the brain, glucose is entirely oxidized to water and CO_2 through the processes of glycolysis, tricarboxylic acid (TCA) cycle and subsequent oxidative phosphorylation. At the end of these processes, 30-36 ATP molecules per glucose are produced. Glucose is essential for the brain because, on one hand, it is its main energy substrate, and on the other hand, it is a fundamental component of glycolipids, glycoproteins and other macromolecules of neural cells. Moreover, glucose takes part in the metabolic pathways leading to the production of glutamate, GABA and acetylcholine, which are the three major neurotransmitters of the brain (Magistretti and Allaman, 2013).

Glycolysis takes place in the cytosol and does not require the presence of oxygen. In this process, one molecule of glucose is metabolized into two molecules of pyruvate. Finally, two molecules of ATP per glucose molecule are produced (figure 1) (Magistretti and Allaman, 2013).

Pyruvate can be further processed, under aerobic conditions, and produces acetyl-CoA that is then linked with oxaloacetate in order to produce citrate. This is the first step of Krebs or TCA cycle that takes place in the mitochondrial matrix and leads to the transfer of three pairs of electrons from nicotinamide adenine dinucleotide (NAD^+) to NADH and one pair of electrons from flavin adenine dinucleotide (FAD) to $FADH_2$. In the Krebs cycle 2 NADH, 1 $FADH_2$ and 1 GTP molecules are produced. Then, the electrons of NADH and $FADH_2$ are transferred to molecular O_2 through the mitochondrial electron transfer chain so as to produce ATP through the process of oxidative phosphorylation which is coupled with ATP synthase (figure 2) (Magistretti and Allaman, 2013).

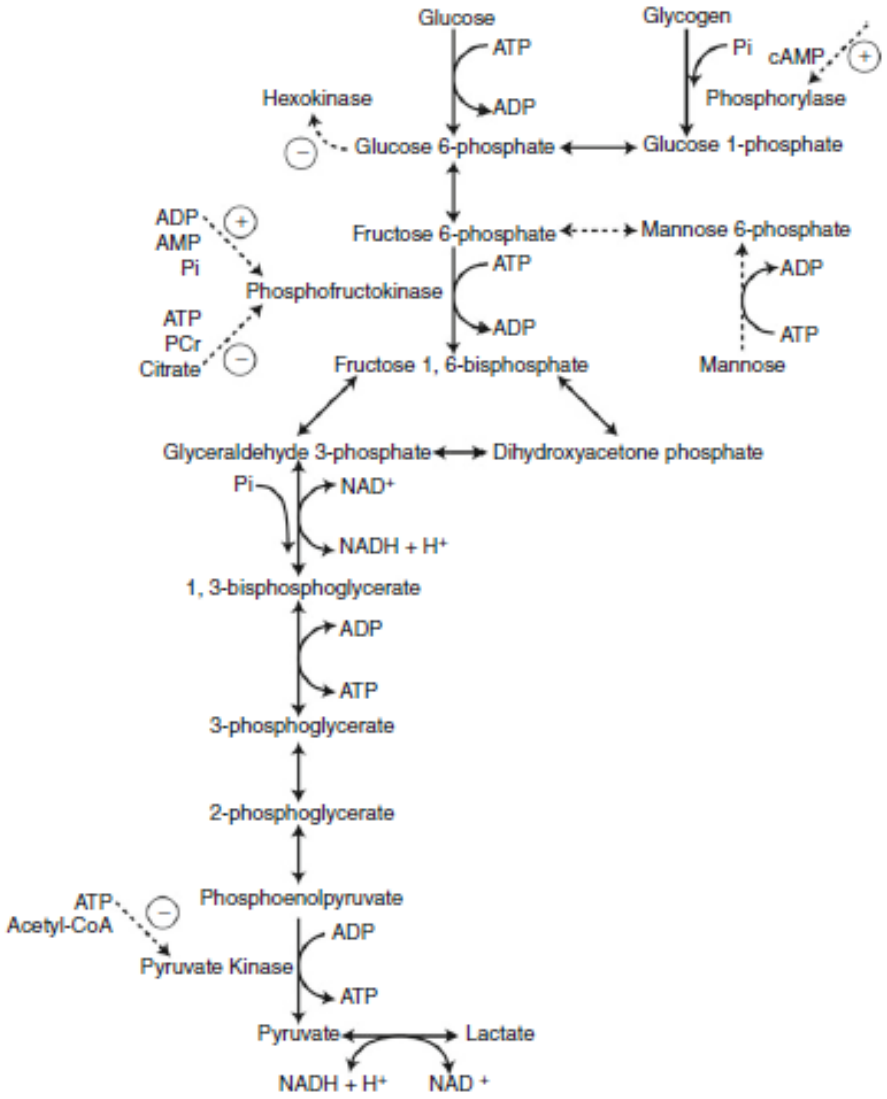

Figure 1: Schematic representation of glycolysis pathway (Magistretti and Allaman, 2013).

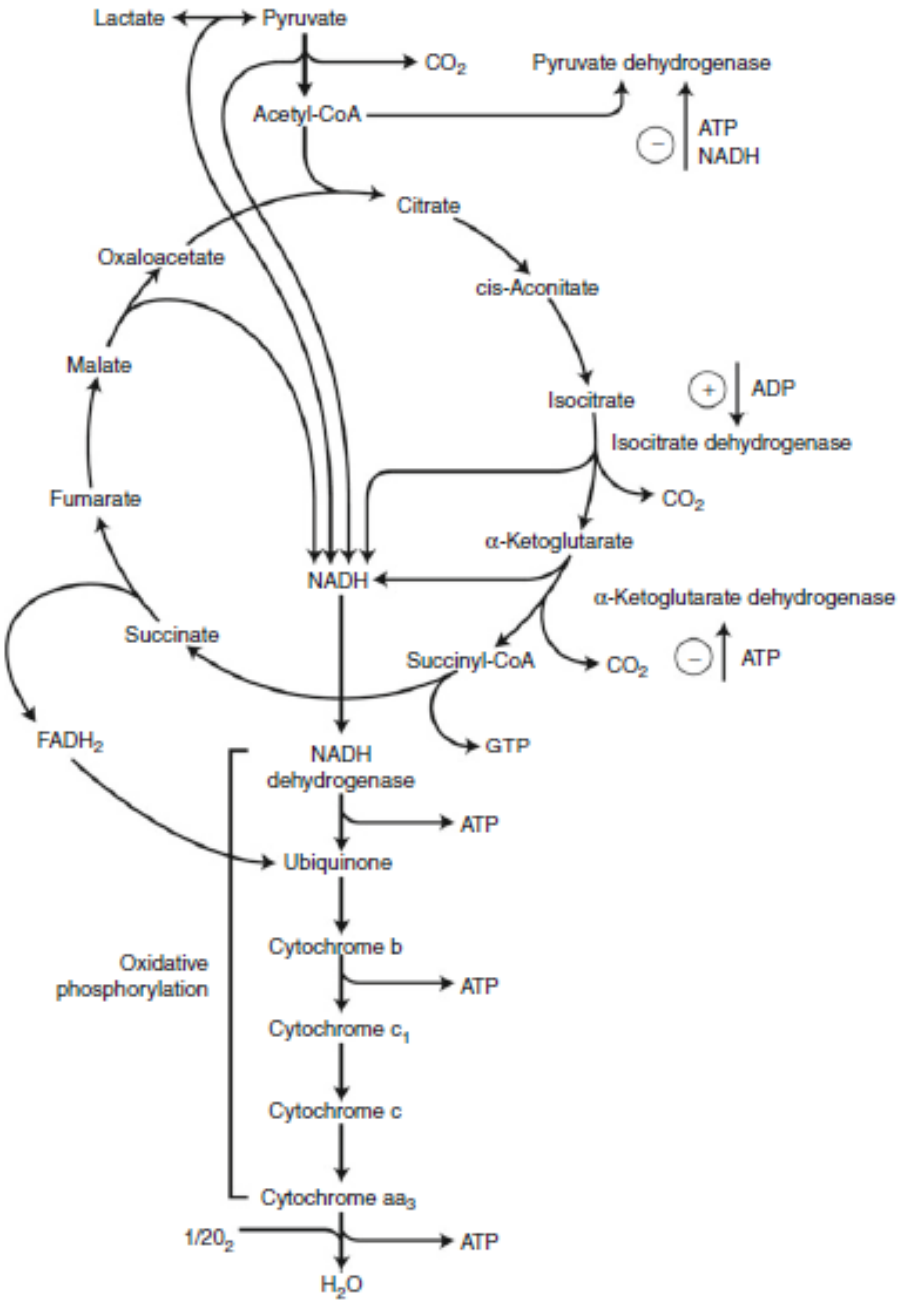

Figure 2: Schematic representation of Krebs or TCA cycle and oxidative phosphorylation (Magistretti and Allaman, 2013).

1.2. Glutamate dehydrogenases (GDHs)

1.2.1. Role of GDHs

Glutamate dehydrogenase (GDH) is an abundant mitochondrial enzyme that catalyzes the reversible interconversion of glutamate into a-ketoglutarate and ammonia, using NADP(H) and/or NAD(H) as cofactors (figure 3) (Hudson and Daniel, 1993). In mammals, GDH is believed to favor the production of a-ketoglutarate in order to be ultimately used for energy production through its participation to the Krebs cycle (McKenna, 2007). Notably, glutamate is the main neurotransmitter of mammals' central nervous system (CNS), while GDH is an enzyme that is important for many cell functions such as Krebs cycle, insulin secretion and ammonia regulation.

The important role of GDH is also indicated by the fact that it is a very abundant enzyme since represents more than 10% of the total amount of proteins that some cells (e.g. liver cells) express in their mitochondrial matrix (Rothe *et al*, 1994).

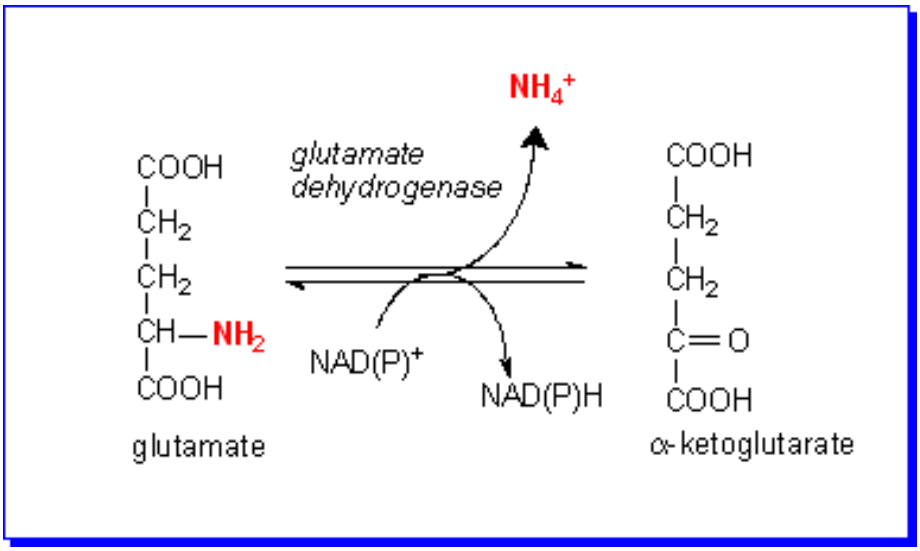

Figure 3: The reversible interconversion of glutamate into a-ketoglutarate and ammonia by the use of GDH.

1.2.2. Regulation of GDHs

GDH is allosterically regulated by several compounds associated with cell metabolism and energy homeostasis including ADP, GTP, ATP, L-leucine and palmitoyl-CoA (Hudson & Daniel, 1993). Among them, GTP acts as inhibitor and ADP as activator of the enzyme depending on cell energy demands. More specifically under circumstances of cellular energy excess, GTP levels rise, thus inactivating GDH and accordingly Krebs cycle is not fed with a-ketoglutarate. On the other hand when energy is diminished; ADP levels are increased leading to activation of GDH which in turn feeds Krebs cycle with a-ketoglutarate. In addition to this, ATP produced as a final step of OxPhos, can also inhibit GDH (although less efficiently than GTP). Furthermore, L-leucine acts as an allosteric regulator of GDH resulting in enhanced activation of the enzyme and shows a synergistic effect when applied with ADP (Shashidharan *et al*, 1997).

1.2.3. Evolution, expression and localization of GDHs

In humans and great apes GDH is encoded by two different genes, *GLUD1* and *GLUD2* with high homology, since it has been proposed that *GLUD2* has emerged after retroposition of *GLUD1* from chromosome 10 to chromosome X. After its genesis, *GLUD2* gene has been evolved through the combination of random mutations and natural selection pressure. The duplication event has been estimated to have happened less than 23 million years ago in the common ancestor of humans and modern apes (Burki and Kaessmann, 2004). Furthermore, this particular retroposition event is unique in the sense that in most cases, an X-linked gene is translocated to an autosomal chromosome. A probable explanation is that this gene movement from chromosome 10 to chromosome X serves the support of some significant events that happen in male spermatogenesis and meiosis (Marques *et al*, 2005). Interestingly, *GLUD2* genesis seems to coincide with brain size increase and functional and structural complexity of the CNS in primates (Burki and Kaessmann, 2004). Apart from abovementioned genes which encode for functional GDH proteins, four *GLUD* pseudogenes have also been found in the human genome. *GLUDP2*, *GLUDP3*,

GLUDP5 localized on chromosome 10, and *GLUDP4* localized on chromosome 18 (Deloukas *et al*, 1993; Plaitakis and Zaganas, 2001).

GLUD1 gene is located in chromosomal location 10q23.3, its length is 44818 base pairs and it is organized into 13 exons and 12 introns, whereas *GLUD2* is an intronless gene located in chromosome X and its length is 2335 base pairs (Michaelidis *et al*, 1993).

GLUD2 has been recently discovered and as a consequence is less studied. In contrast to *GLUD1*, that is expressed ubiquitously, *GLUD2* is mainly expressed in the retina, frontal, pariental and temporal lobes of the brain, kidney and testis (Shashidharan *et al*, 1994). In the testis, *GLUD2* is expressed in Sertoli cells, while in the brain it is mainly expressed in astrocytes rather than neurons according to immunohistochemical studies (Spanaki *et al*, 2010; Zaganas *et al*, 2012).

The expression of *GLUD2* in astrocytes might be connected to the neuronal supportive and nourishing role of these cells. Astrocytes can eliminate glutamate excitoxicity by uptake of the excess glutamate from the synaptic cleft, after neurotransmission. The abundant glutamate is then conversed to glutamine, which is a more stable compound, by the action of glutamine synthetase. Glutamine is in turn transported to neighboring neurons, where is again conversed to glutamate depending on cell energy or neurotransmission demands. In this way, astrocytes ensure that neurons will obtain the amount of glutamate or glutamine needed each time contributing in this way into energy requirements (Zaganas *et al*, 2012).

GDH has been detected in the mitochondrial matrix by several studies (Rosso *et al*, 2008; Mastorodemos *et al*, 2009,). This is not surprising since mitochondria also host Krebs cycle and OxPhos procedures. The transport of GDH into the mitochondrial matrix is achieved through its leader peptide, occupying the first 53 amino acids of the protein. Specifically, the leader peptide is cleaved when GDH enters the mitochondrial membrane and in this way the mature enzyme it leaded into the mitochondrial matrix. where it is ultimately functional. The importance of the leader peptide for organelle targeting is indicated by the fact that its removal results in GDH accumulation at the cytosol (Mastorodemos *et al*, 2009).

1.2.4. Functional differences between GDH1 and GDH2

The functional human GDH isoproteins differ only in 15 out of their 505 amino acids which represent the 4% of their total length. However, they display significantly different regulation patterns, thermal stability and optimal function conditions (Kanavouras *et al*, 2007). More specifically, hGDH1 is heat stable, sensitive to GTP inhibition and maintains 40% of its maximal activity even in the absence of allosteric activators (ADP). On the other hand, hGDH2 is heat-labile, resistant to GTP inhibition and retains a basal activity which represents 2-8% of its maximal activity (Plaitakis *et al*, 2000; Shashidharan *et al*, 1997). GDH2 is more responsive to activation by ADP and/or L-leucine compared to GDH1. Nevertheless, hGDH2 binds ADP with lower affinity than hGDH1 while its affinity of L-leucine is similar to GDH1's (Kanavouras *et al*, 2007). Furthermore, the two enzymes also display differential inhibition by estrogens, polyamines and neuroleptics (Zaganas *et al*, 2014). More specifically, hGDH2 is inhibited by palmitoyl-CoA, spermidine, ECGC and neuroleptics more efficiently than hGDH1 (Choi *et al*, 2007; Plaitakis *et al*, 2011; Spanaki *et al*, 2012). Finally, human GDHs also differ in their optimum pH, with hGDH1 operating better at pH 8 while hGDH2 at pH 7.5 (Kanavouras *et al*, 2007).

Site-directed mutagenesis on *GLUD1* gene revealed that substitution of two out of the 15 amino acids that differ between GDH1 and GDH2 are responsible for most of the new properties that GDH2 acquired. Specifically, Ala for Gly456 made GDH2 resistant to GTP inhibition without affecting its activation by ADP and L-leucine (Zaganas and Plaitakis, 2002), while Ser for Arg443 accounts for the low basal activity, heat lability and estrogen sensitivity of hGDH2 (Zaganas *et al*, 2002). The combined action of these mutations led to a GDH enzyme showing similar, but not identical properties with hGDH2 (Kanavouras *et al*, 2007). Hence, other aminoacid substitutions probably account for the special properties that hGDH2 acquired after *GLUD2* establishment..

The need for a second, tissue-specific GDH enzyme displays an alternative regulation pattern, might reflect special needs of the tissues where it is expressed. For instance, hGDH2 might have been evolved to function even under conditions of high energy load (high GTP concentrations), as a response to elevated glutamate levels which are present in synapses after neuronal

activation, thus preventing glutamate excitotoxicity (Zaganas *et al*, 2014). The activation of hGDH2 by ADP, on the other hand, maybe was chosen in order to regulate the glutamate flux. So, when energy is depleted and therefore ADP/ATP ratio is high, GDH2 is activated so as to provide energy to the cell (Plaitakis and Zaganas, 2001; Schousboe *et al*, 2011). Intensive activation by L-leucine can be an additional fine tuning mechanism that renders hGDH2 more sensitive to ADP regulation, in order to respond to even subtle changes of the cell energy status (Plaitakis *et al*, 2003).

1.2.5. Tertiary structure of GDHs

As far as their tertiary structure is concerned, human GDHs are composed of six identical subunits. Each subunit consists of 505 amino acids, has a molecular weight of 56 kDa and is composed of three main domains, namely the NAD^+ binding domain, the glutamate binding domain and the regulatory domain. The first domain is essential for the reaction catalysis, the glutamate binding domain is important for the core formation of the hexamer and the regulatory domain is composed of the antenna region and the pivot helix. Antenna is the part that protrudes from each subunit and consists of a helix and a random coil (figure 4). It seems to be involved in allosteric regulation, cooperativity of the subunits and catalytic efficiency of GDH (Zaganas *et al*, 2014).

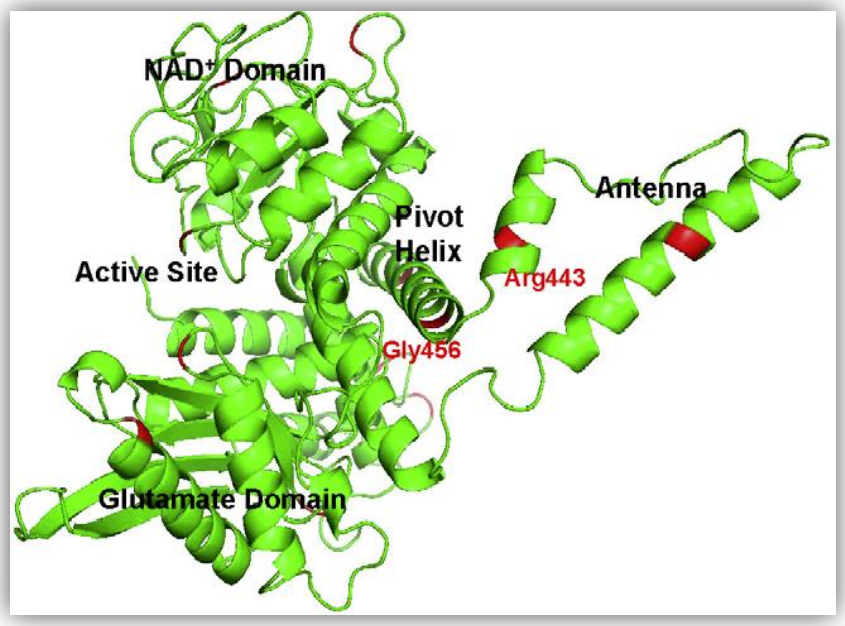

Figure 4: hGDH1 structural model. Major amino acid residues that are different between hGDH1 and hGDH2 are highlighted in red (Zaganas *et al*, 2009).

1.2.6. Role of GDHs in signaling

hGDH is also involved in Serine/Threonine kinase mammalian Target of Rapamycin Complex 1 (mTORC1)signaling. mTORC1 is composed of mTOR, Raptor and mLST8 and it is activated by several aminoacids, growth factors and energy indices. Its role is to ensure that the cell will acquire the essential nutrients in order to be proliferated (Yecies and Manning, 2011). Mechanistically, mTORC1 promotes the mediated by the proteasome, degradation of cAMP response element binding-2 (CREB2). CREB degradation is probably caused due to the binding of the E3 ligase and beta-transducin repeat containing E3 ubiquitin protein ligase (βTrCP) to CREB2. As a result mTORC1 represses the transcription of SIRT4. SIRT4 is a NAD-dependent sirtuin, localized in mitochondria, that inhibits GDH. In this way, a-ketoglutarate cannot be produced anymore (figure 5). However, it is not clear yet how exactly GDH is involved in signaling (Csibi *et al*, 2013).

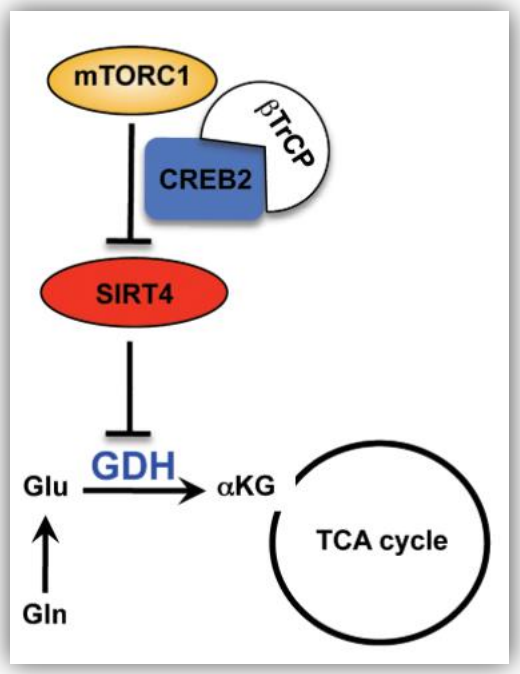

Figure 5: Role of GDH in mTORC1 signaling (Csibi *et al*, 2013).

1.2.7. Involvement of GDHs in human diseases

Mutations in GDH have been associated with several diseases. However, in all these cases the function of GDH is not completely abolished, maybe because it is a crucial enzyme and total loss of its function might lead to death.

A gain of function mutation of hGDH2 has been related with accelerated age at onset of Parkinson's disease. Parkinson's disease is a multifactorial disease that has been associated with mitochondrial dysfunction. The researchers found that the T1492G variant, which causes the substitution of Ala for Ser445, leads to acceleration in Parkinson's disease age at onset of about 6-13 years in hemizygous men. The effect of this mutation is the reduction of hGDH2 sensitivity for GTP inhibition and the increased basal activity of protein. In addition to this, it also causes an increase of the sensitivity that GDH has in estrogen suppression. This can explain the reason why women who are heterozygous for this mutation do not present similar disease acceleration. Possibly, mutated hGDH2can lead to uncontrolled oxidation of glutamate, thus inducing energy production (through TCA cycle and OxPhos) and subsequent elevation of ROS (Plaitakis *et al*, 2010).

In addition to this, hyperinsulinism– hyperammonemia (HI-HA) syndrome has been attributed to dominant mutations in *GLUD1* gene. Patients that suffer from this syndrome display hypoglycemia and epileptic seizures after a protein rich meal. In HI-HA syndrome, several mutations in *GLUD1* gene weaken GTP inhibition, leading to an over reactive GDH1. As a result, the amount of a-ketoglutarate that enters the Krebs cycle is increased which subsequently causes an increase in ATP levels and leads to excessive insulin release by the β-pancreatic cells (figure 6) (Stanley *et al*, 1998).

Moreover, several studies support that deregulation of glutamate homeostasis is related with age progressive disorders, such as Alzheimer's disease (Olabarria *et al*, 2011; Kulijewicz-Nawrot *et al*, 2013).

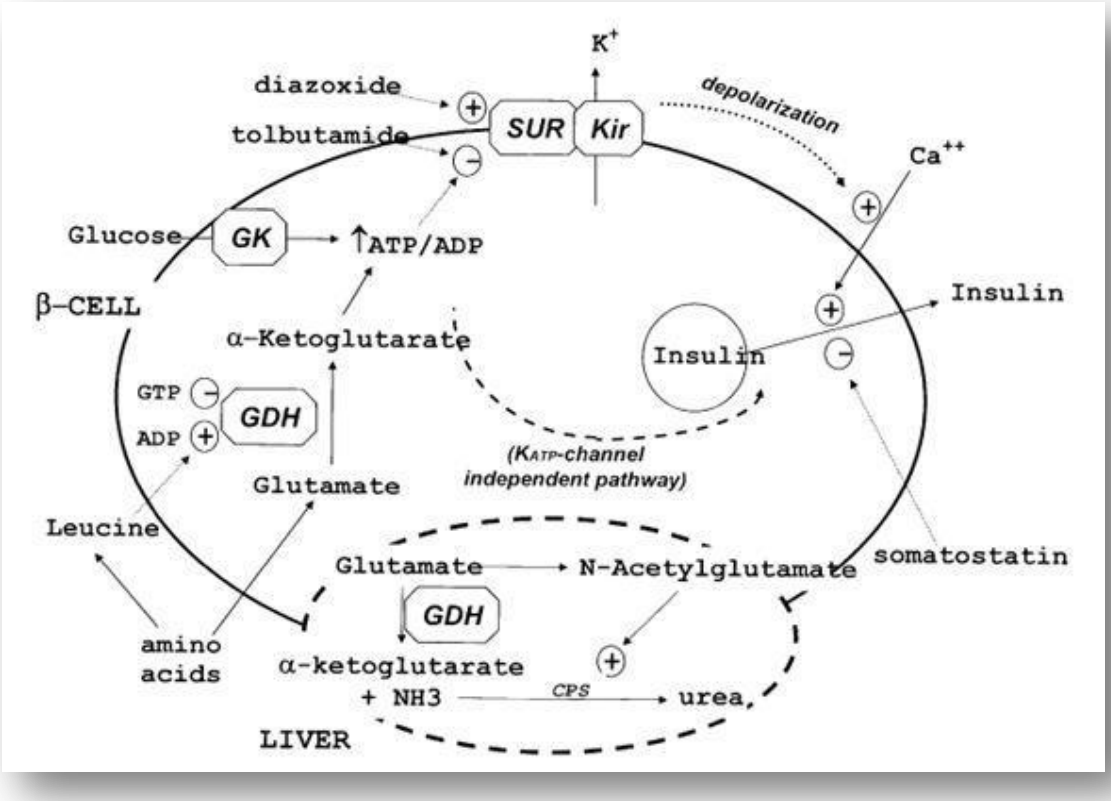

Figure 6: The cascade of events leading to HI/HA syndrome (Stanley, 2009).

1.2.8. Studies of GDH deletion

GDH is an enzyme that has been studied extensively but surprisingly only few researchers attempted to inhibit its expression. First of all, the deletion of GDH1 in β-cells abrogated part of the insulin secretory response. Insulin is a hormone that is produced by β-cells and it is crucial for glucose homeostasis. Insulin exocytosis is triggered by an augmentation in the calcium that β-cells contain in their cytoplasm. This increase in cytosolic calcium occurs after the administration of nutrients (Henquin, 2000). GDH is thought to play a role in secretary response development. For this reason, researchers generated transgenic mice that had GDH1 deleted particularly in β-cells. They observed that these mice could still secrete insulin, but in a lower rate. So, they concluded that GDH1 is not essential for glucose recognition since insulin secretion could begin even without it, but the amplitude was significantly limited (Carobbio *et al*, 2009).

Furthermore, loss of GDH1 in the central nervous system of mice altered glutamate handling without affecting the synaptic transmission. More specifically, the researchers created mice that had GDH1 deleted particularly in the CNS. These mice were viable, could obtain offsprings and did not have any behavioral deficits. The researchers observed that in GDH1 knockout mice the activity of the enzyme was inhibited in both directions (oxidative deamination and reductive amination). However, no change in glutamate levels in comparison with the wild type animals was observed (Frigerio *et al*, 2012).

1.3. Description of the CRISPR/Cas system

CRISPR-Cas is a system that it is normally used by bacteria and other microbes as a protection mechanism against invading DNA (Horvath and Barrangou, 2010). The best characterized CRISPR system is the type II. In this system, the host genome harbors repeat sequences among which the invading DNA is incorporated. CRISPR repeat arrays are transcribed and processed into CRISPR RNAs (crRNAs). Each of them contains a variable sequence transcribed from the invading DNA, which is known as “photospacer” sequence, and a piece of the repeated sequence. Each crRNA hybridizes with a transactivating CRISPR

RNA (tracrRNA) that is recognized by Cas9 nuclease that also associates with the complex (Deltcheva *et al*, 2011). The "photospacer" encoded sequence leads Cas9 to recognize and cleave complementary target DNA sequences as long as they are adjacent to a photospacer motif (PAM) sequence (figure 7).

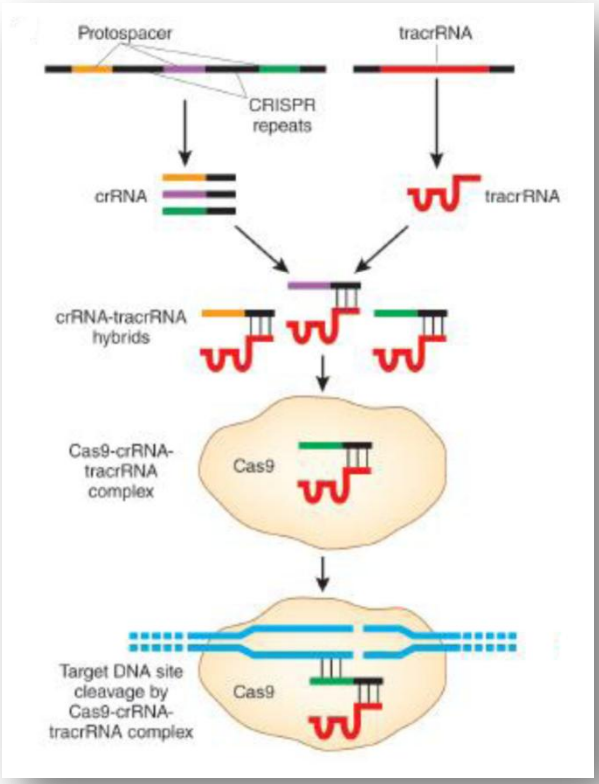

Figure 7: Naturally occurring Type II CRISPR-Cas9 system (Sander and Joung, 2014).

This naturally occurring CRISPR-Cas system has been adapted in order to be used for targeted genome engineering (Jinek *et al*, 2012). In this case, the Cas9 nuclease and a guide RNA (gRNA), that recognizes the sequence of interest, are required. The gRNA contains 20 nucleotides at the 5' end that correspond to the "photospacer" sequence of the crRNA of the naturally occurring CRISPR-Cas9 system and it is upstream of a PAM sequence at the genomic recognition site.

1.4. Aim of the study

Aim of the current study is to examine the effect of *GLUD*1/2 elimination in human cell cultures, using the CRISPR/Cas9 system, a new potent method that is increasingly employed in cell research. Specifically, the current study focuses on the design of *GLUD1* and *GLUD2* specific sgRNAs and incorporation of these in plasmids carrying sequences required for the CRISPR/Cas9 system. The resulting plasmid constructs are currently used to transfect HEK293 mammalian cell lines in order to disrupt hGDH1 and/or hGDH2 expression.

The reason we are interested in these proteins is that they are crucial for energy metabolism and other cell functions; yet, the impact of their deletion has not been studied extensively. So far, knock out studies in mice of glutamate dehydrogenases focused on hGDH1 and not hGDH2 (Carobbio *et al*, 2009; Frigerio *et al*, 2012) , given that the mice do not possess hGDH2, and have been so far inconclusive, since it is possible that the mice develop compensatory mechanisms through development. However, the CRISPR/Cas system was chosen since it seems to be a very promising technique for the generation and study of gene deletions that occur abruptly, enabling to study the effect of gene function loss without elaborate compensatory mechanisms. In the near future, cellular viability and mortality rate will be estimated in the hGDH1/2 deficient cells we have generated. Interestingly, in parallel experiments in our lab, overexpression of hGDH1 and hGDH2 led to increased cellular viability in cultures of stable lines HEK293 in comparison with the wild type cell lines (Mathioudakis *et al*, unpublished data). Furthermore, potential metabolic changes brought about by the absence of glutamate dehydrogenases will be examined.

2. Materials and Methods

Overview

The overview of the cloning method is the following:

i. Annealing and phosphorylation of the oligos

ii. Digestion using BbSI enzyme
iii. Ligation reaction
iv. Transformation of competent DH5A *E.coli* cells
v. Bacterial colony selection
vi. Mini-prep isolation of high-copy plasmid DNA from *E.coli* cells
vii. Sequencing
viii. Midi-prep isolation of high-copy plasmid DNA from *E.coli* cells
ix. Cell culture
x. Bradford assay
xi. Western blot

2.1. Annealing and phosphorylation of the oligos

Materials

- sgRNA 24-mer oligos (100 μM)
- sgRNA 24-mer reverse complement oligos (100 μM)
- 10x T4 ligation buffer
- T4 Polynucleotide Kinase (10,000 U/ml)
- ddH_2O

SgRNA 24-mer oligos

SgRNAs were designed using the "CRISPR direct" online tool and were ordered from Macrogen Europe. Each of them, consist of a 20-mer nucleotide sequence that corresponds to the "photospacer sequence" and it is upstream of the PAM sequence at the genomic recognition site. "CRISPR direct" online tool proposed many possible sgRNAs. From them sgRNAs located more closely to the 5' end where chosen. Furthermore, this tool ensured the avoidance of off-target effects of Cas9 that is, preventing cuts at other, non target sites in the genome. Two sgRNAs were designed for each *GLUD* gene so as to increase the efficiency of GDH deletion. In addition to this, sgRNAs designed for *GLUD1* knockout differed in one or two nucleotides from those designed for *GLUD2* knockout in order to target specifically the corresponding gene. A "CACC" sequence was added before the 20-mer guide sequence and a "AAAC" sequence before the guide's reverse

complement sequence for cloning into the pX458 plasmid vector using BbsI restriction enzyme (Table 1).

Gene	Name	Sequence	Position
GLUD1	*Glud1*A_KO_S	5' CACC-GGGCTCGGCGTCCGCCGACT 3'	57-79
	*Glud1*A_KO_AS	5' AAAC-AGTCGGCGGACGCCGAGCCC 3'	
	*Glud1B*_KO_S	5' CACC-TGAGCCGGCGCAACGACCCG 3'	71-93
	*Glud1B*_KO_AS	5' AAAC-CGGGTCGTTGCGCCGGCTCA 3'	
GLUD2	*Glud2A*_KO_S	5' CACC-GAGCCGGCGCAACGACCCGG 3'	72-94
	*Glud2A*_KO_AS	5' AAAC-CCGGGTCGTTGCGCCGGCTC 3'	
	*Glud2B*_KO_S	5' CACC-CTCGCAGCCGGGGCTCGCAT 3'	120-142
	*Glud2B*_KO_AS	5' AAAC-ATGCGAGCCCCGGCTGCGAG 3'	

Table 1: Protospacer sequences and their reverse complements with "CACC" and "AAAC" added for cloning into the pX458 vector using BbsI restriction enzyme.

Procedure

The monoclonal sgRNAs have to be annealed in order to form double-stranded oligonucleotides. Furthermore, phosphorylation of the 5' end is essential in order to permit the following ligation reaction. So, the materials are introduced into PCR tubes and the reactions take place in a thermocycler.

Program

- 37 °C for 30 minutes
- 95 °C for 5 minutes
- cooling down to 25 °C with a rate of 5 °C/minute

2.2. Digestion using restriction enzymes

Restriction enzymes are naturally occurring enzymes that recognize and cleave DNA sequences at specific sites leaving protruding single stranded tails at each end. So, cleavage of the desired plasmid vector and insert with the same restriction enzymes create complementary ends that permit their subsequent ligation.

Materials

- pX458 circular plasmid vector (100 ng)

- BbSI restriction enzyme
- 10x enzyme specific buffer G
- ddH_2O

Procedure

All the materials are placed in a tube and the sample is firstly incubated at 37^oC for 1 hour, where BbSI enzyme optimally functions, and then at 80^oC for 20 minutes in order to deactivate the enzyme.

2.3. Ligation reaction

Ligation reaction uses ligase to connect endonuclease-digested vectors with similarly digested inserts. Normally ligase is an enzyme that identifies and corrects breaks in DNA by catalyzing the formation of phosphodiester bonds between the 3'hydroxyl and 5'phosphate groups of the DNA backbone. In this case, T4 ligase that originates from *E.coli* bacterial strain was used. From the ligation reaction, a recombinant vector is created which is subsequently used to transform bacterial cultures, where it can be replicated. An example of a ligation reaction of pX458 vector and an oligo is depicted in figure 8.

Materials

- pX458 circular plasmid vector (100 ng)
- annealed oligos (1 μm)
- T4 DNA ligase
- 10x T4 DNA ligase buffer
- ddH_2O

Procedure

The DNA of both the plasmid vector and each annealed oligonucleotide are placed into a tube that also contains 10x buffer, T4 DNA ligase and ddH_2O up to the desired total volume. Then the samples are incubated at 16^oC overnight.

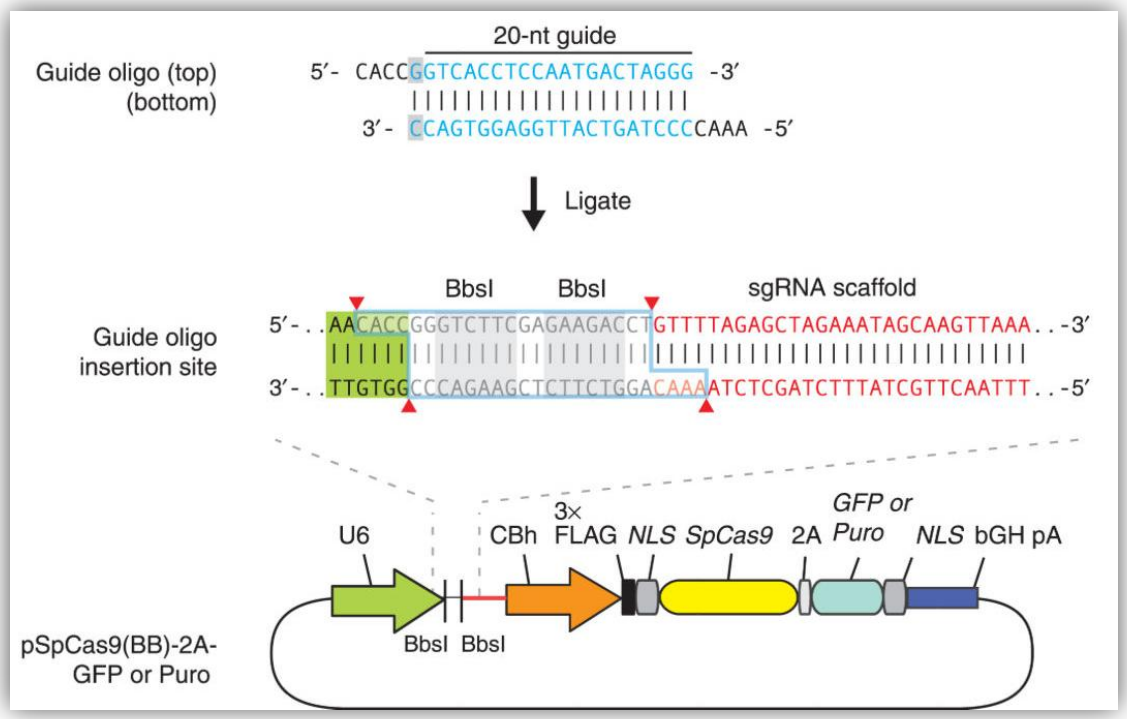

Figure 8: Schematic representation of cloning of the annealed oligos into pX458 plasmid vector. The oligos contain protruding ends for ligation into the pX458 vector cut with BbSI. Digestion of pX458 with BbSI permits the replacement of BbSI restriction sites with oligos sequences.

2.4. Transformation of bacterial cells

Transformation is the process by which foreign DNA is introduced into cells. For this purpose competent bacterial cells are used.

Materials

- competent DH5A *E. coli* cells
- LB medium
- recombinant pX458 plasmid vector
- agar plates

Preparation of agar plates

For the preparation of agar plates, 1.2 g agar is introduced into 100 ml LB and autoclaved for 20 minutes at 121° C. The agar solution is allowed to cool at approximately 55°C, before selective antibiotics are added (100μl ampicillin (100μg/ml)). Finally, the solution is poured in Petri plates.

Preparation of LB medium

For the preparation of 500 ml LB medium 5 g Bacto-tryptone, 2.5 g Yeast extract and 5 g NaCl are dissolved into 500 ml ddH_20. Then, the pH of the mixture is adjusted to 7 and the solution is autoclaved for 20 minutes at 121^oC.

Preparation of *E.coli* electrocompetent cells

Firstly, a small amount of frozen glycerol stock of DH5A bacterial cells is inoculated into 2 ml LB and incubated at 37 ^oC overnight. Then, 400 μl from the starter culture are inoculated into 150 ml LB* (5 g Bacto-tryptone, 2.5 g Yeast extract and 2.5 g NaCl, 500 ml ddH_20) and the culture grows at a 37 ^oC shaking incubator until the OD_{600} be equal to 0.65. After that, the culture is centrifuged for 15 minutes at 4000 rpm at 4 ^oC. Then, the supernatant is removed and the pellet is resuspended in 100 ml ice cold sterile ddH_20. Then, the samples are centrifuged for 15 minutes at 4000 rpm at 4 ^oC and after the removal of the supernatant; the pellet is resuspended in 50ml ice cold dH_20. This process is repeated three more times with the resuspension of the pellet in 10 ml, 5 ml and 1 ml dH_20 each time. Finally, the pellet is resuspended in 500μl 10% glycerol and 100μl aliquots are prepared and stored at -80 ^oC.

Procedure

Initially, agar plates are placed in the 37^oC incubator. Plasmid DNA is mixed with the competent cells; they are placed into an electroporation cuvette and treated with 1350V current for 5 milliseconds in order to permit the introduction of the recombinant vector into the *E.coli* cells. Cells are subsequently grown in 1 mL LB medium at a 37^oC shaking incubator for 1 hour. Then, the cell sample is centrifuged for 1 minute at 11000 g at room temperature, most of the supernatant's volume is discarded and the pellet is resuspended with the remaining. Finally, we plate the cells on agar medium that contains the appropriate antibiotics and we incubate them at 37^oC overnight.

2.5. Bacterial colony selection

Materials

- LB medium
- ampicillin (100μg/ml)

Procedure

The desired bacterial colonies (presumably all the colonies arisen in a plate containing selective antibiotics are expected to have received the recombinant plasmid) are chosen and picked from the plates, using a sterile pipette tip. The tip is then placed in 3 ml LB with ampicillin, and the culture is incubated in a 37^{o}C shaking incubator overnight.

2.6. Mini-prep isolation of high-copy plasmid DNA from E.coli cells

Materials

- Plasmid DNA purification kit (Macherey-Nagel, Deutschland)

Procedure

Initially, *E.coli* cells are centrifuged for 30 seconds at 11000 g at room temperature. Then, the supernatant is removed and the pellet is resuspended in 250 μl of Buffer A1. After that 250 μl of Buffer A2 is added and mixed gently by inverting the tube 8 times. The mixture is incubated at room temperature for no more than 5 minutes before the subsequent addition of 300 μl Buffer A3. Then, centrifugation for 5 minutes at 11000 g at room temperature occurs in order to permit the clarification of the lysate. After that the supernatant is transferred into a DNA binding column and is centrifuged for 1 minute at 11000 g at room temperature. The flow-through is discarded, 500 μl Buffer AW is added and be centrifuged for 1 minute at 11000 g at room temperature. After the removal of the flow-through Buffer A4 is added and the sample is centrifuged for 1 minute at 11000 g at room temperature. A centrifugation with an empty tube for 2 minutes at 11000 g at room temperature follows in order to ensure the total removal of the ethanol that A4 contains. Then, the addition of Buffer AE permits the elution of the DNA after a centrifugation for 1 minute at 11000 g.

2.7. Sequencing analysis

After the isolation of the recombinant plasmid from bacterial cells, a small amount was sent to Macrogen for sequencing analysis, to ensure that each plasmid contains the respective desired insert. Sequencing was performed using U6 promoter forward primer: CGTAACTTGAAAGTATTTCGATTTCTTGGC. This primer was chosen because pX458 plasmid vector uses the human U6 promoter to drive gene expression.

2.8. Midi-prep isolation of high-copy plasmid DNA from E.coli cells

A sequence-verified colony from each construct was chosen and inoculated into a larger culture volume for large scale plasmid extraction.

Materials

- Plasmid DNA purification kit (Macherey-Nagel, Deutschland)

Procedure

3–5 mL starter culture in LB medium containing ampicillin is inoculated with a single colony picked from a freshly streaked agar plate and is incubated in a 37^{o}C shaking incubator overnight. Then, 150 μl of the grown culture are added in 100ml LB medium containing ampicillin and the culture is incubated again in a 37^{o}C shaking incubator overnight. After that the samples are centrifuged for 15 minutes at 6000 g at 4 ^{o}C, the supernatant is discarded and the pellet is resyspended in 8 ml Resuspension Buffer RES. Then, 8 ml Lysis Buffer LYS are added to the suspension, the tube is inverted 8 times and incubated at room temperature for 5 minutes. Meanwhile, a DNA binding column is equilibrated by applying 12 ml Equilibration Buffer (EQU) onto the rim of the column filter. After that 8 ml of Neutralization Buffer (NEU) are added to the suspension of the cell lysate, the tube is inverted 8 times and centrifuged for 15 minutes at 6000 g at room temperature. The resulting supernatant is loaded onto the column and the column is washed by adding 5 ml Equilibration Buffer (EQU). Subsequently, the filter is removed and a second wash with 8 ml of Buffer WASH follows. After that

the plasmid DNA is eluted with 5 ml Elution Buffer (ELU) which is preheated at 50 ^{o}C. Then, 3.5 ml frozen isopropanol is added to the elution to precipitate the plasmid DNA and the sample is centrifuged at 15000 g for 30 minutes at 4 ^{o}C. Washing of the sample with 2 ml of 70% ethanol solution follows, before a last centrifugation at 15000 g for 5 minutes at room temperature. Eventually, ethanol is completely removed and when the pellet has been drought it is dissolved in dd$H_2$0.

2.9. Cell culture

Materials

- freezing medium (60% DMEM plain, 30% heat inactivated FBS, 10% DMSO)
- DMEM growth medium (445 ml DMEM Base, 50 ml heat inactivated FBS, 5 ml Penicillin/Streptomycin)
- Lysis Buffer H (Buffer H, 1% triton, protease inhibitor 1x)
- 100x protease inhibitor

Freezing of HEK293 cells

Cells are detached from their growth flask and the cell suspension is centrifuged at 1200 rpm for 10 minutes at room temperature. Then, the supernatant is discarded and the pellet is resuspended in 1ml freezing medium. FBS is a serum-supplement and DMSO is a cryoprotective agent that is essential for reducing the freezing point of the medium and also allows a slower cooling rate, reducing the risk of ice crystal formation, which can damage cells and cause cell death. The cell suspension is put in suitable cryovials which can be stored at -80°C for six months or for years in liquid nitrogen vessels.

Thawing of frozen HEK293 cells

A cryovial that contains the frozen cells is removed from -80 ^{o}C and is quickly placed into a 37°C water bath. Cells are thawed quickly by shaking the cryovial in the waterbath. The suspension is transferred into a falcon containing complete DMEM growth medium and is centrifuged at 1200 rpm for 10 minutes at room temperature. The supernatant is discarded and the cells are resuspended in fresh

complete growth medium. Finally, the resuspension is placed in a 25 cm^{-2} cell culture flask and the cells are grown in a 37°C incubator supplemented with CO_2 and water vapor.

Preparation of whole HEK293 Lysate

The cell suspension is centrifuged at 1500 rpm for 10 minutes at room temperature so as to obtain a cell pellet. Then, the pellet is resuspended in 300 μl Lysis Buffer H that permits cell lysis. 1x protease inhibitor cocktail addition ensures cell lysate protein stability. The lysate is cooled for 30 minutes on ice, and is subsequently centrifuged at 10000 rpm for 10 minutes at 4°C. After the centrifugation, the supernatant that contains the proteins of the cells is collected and stored at -80°C.

2.10. Bradford assay

Bradford assay is a fast and reliable procedure used to measure protein concentration in a solution. It is a colorimetric protein assay, based on the binding of the Bradford dye to proteins; as the protein concentration increases, the color of the test sample shifts from reddish-brown to blue. In parallel, the absorbance changes from 470 nm to 595 nm.

Materials

- cell lysate
- Bradford reagent (Biorad)
- ddH_20

Procedure

First of all, 1 μl of the protein lysate is dissolved in 799 μl ddH_20. Then, 200 μl of the Bradford reagent is added to the solution and the mixture is incubated for 5 minutes at room temperature. Then, the absorbance at 595 nm is measured spectrophotometrically. Protein quantification is achieved by using a standard curve obtained from measurement of different BSA concentrations.

2.11. Western blot

Western blotting is a technique through which proteins can be identified with the use of specific antibodies that recognize them. Before that proteins have been separated from one another based on their molecular weight by SDS gel electrophoresis.

Materials

- resolving gel 10% (4.1 ml ddH_20, 3.3 ml 30% bis/acrylamide, 2.5 ml gel buffer *1.5 Tris-HCl ph 8.8, 100 μl 10% SDS, 50 μl 10% APS, 5 μl TEMED)
- stacking gel 4% (6.1 ml ddH_20, 1.3 ml 30% bis/acrylamide, 2.5 ml gel buffer *0.5 Tris-HCl ph 6.8, 100 μl 10% SDS, 50 μl 10% APS, 10 μl TEMED)
- 1x running buffer (For 10x running buffer: 30.3 g Tris base, 144 g glycine, 10 g SDS, 1000 ml ddH_20)
- ladder
- 1x loading buffer
- 4x loading buffer
- wattman pieces (5 cm * 9 cm)
- nitrocellulose membrane (6 cm * 9 cm)
- transfer buffer (100 ml 10x running buffer, 700 ml ddH_2O, 200 ml MethOH)
- 5% blocking solution (2.5 g milk powder, 50 ml 1x PBS-tween)
- primary antibody diluted in 5% milk
- 1x PBS
- 1x PBS-tween
- secondary antibody
- ECL A and B reagents

Gel preparation

The resolving gel is prepared by combining all reagents except of APS and TEMED which are added in the very end, as these start the polymerization of acrylamide immediately. The mix is poured between two glasses that are put together on a standing apparatus, leaving 1.5 mm space between them. After the gel mix is poured, it is overlaid with isopropanol so as to not dry and is left to

polymerize. Subsequently, the stacking gel is prepared and it is placed above the resolving gel. Then, the combs are inserted and the stacking gel is allowed to be polymerized.

Sample preparation

1x loading buffer and 4x loading dye that also contains 4% mercaptoethanol, that breaks disulfite bonds, are added to each sample. That permits us to load the samples properly into the gel wells, avoiding their diffusion in the running buffer, while it also offers a rough estimation of how far the samples have run. Then, the samples are heated at 100^{o}C for 5 minutes. Thus, proteins are denaturated so as to get accessible by the first antibody that will be added later.

SDS PAGE

Meanwhile, the inner chamber of the gel electrophoresis apparatus as well as the rest of the apparatus, are filled with 1x running buffer. The samples are loaded into the gel's wells and are electrophoresed at 100V for approximately 30 minutes in order to run evenly by the end of the stacking gel and then at 150V for another 1.5 hour.

Transfer

After that the transfer apparatus cassette is placed in a tray containing cold transfer buffer in which wattmann papers, sponges and a nitrocellulose membrane are soaked. The gel is removed from the glasses and the stacking gel is discarded. Subsequently, a sponge, three pieces of filter paper, the gel, the membrane, three more pieces of filter paper and one more sponge are sequentially placed on the black side of the cassette. In this way, the gel and the membrane are enclosed between absorbent materials, and the cassette is clamped between solid materials so as to maintain tight contact between the gel and membrane that will help the transfer of protein from the former to the latter. Then the cassette together with ice packs, is placed in the transfer apparatus, the container is filled with ice-cold transfer buffer and runs at 310 mA for 1 hour.

Afterwards the membrane is removed from the cassette and it is soaked in 5% blocking solution before it is incubated at room temperature for 1 hour on a shaking machine. Blocking prevents non-specific background binding of the

primary and secondary antibodies to the membrane proteins. Then, the blocking solution is removed and the membrane is incubated with the specific for each protein primary antibody diluted 1:4000 in 5% milk on a shaker at 4 ^{o}C overnight. The next day the first antibody is removed and the membrane is firstly washed three times with 1x PBS quickly, then three washes of 15, 5 and 5 minutes with PBS-tween are performed respectively. The second antibody is applied on the membrane and they are incubated at room temperature for 1 hour under agitation. The second antibody is discarded and three quick washes with 1x PBS, three washes of 15, 5 and 5 minutes with PBS-tween and two more washes of 5 minutes with 1x PBS are performed.

Then the membrane is placed on a flat surface and equal amounts of ECL A and B reagents are added dropwise and incubated for 5 minutes at room temperature. Finally, the membrane is exposed on a film in the dark room and the proteins are visible.

2.12. Transfection of HEK293 mammalian cell lines with the recombinant plasmid vectors

HEK293 mammalian cell lines are transfected with plasmids carrying the sgRNAs that target *GLUD1* and *GLUD2* genes. The transfection with the recombinant plasmids is performed in four different combinations. Transfection of mammalian cells with pX458-*Glud1A* and pX458-*Glud1B* plasmid vectors is performed for the specific knockout of *GLUD1* gene. Deletion of GDH2 expression specifically will be achieved by the transfection of mammalian cell lines with both pX458-*Glud2A* and pX458-*Glud2B* plasmid vectors. pX458-*Glud1A* plasmid vector that targets *GLUD1* gene more specifically compared to pX458-*Glud1B* plasmid vector, together with pX458-*Glud2A* plasmid vector that targets *GLUD2* gene with higher efficient compared to pX458-*Glud2B* plasmid vector, is used for the knockout of both *GLUD1* and *GLUD2* genes. In addition to this, transfection of HEK293 mammalian cell lines with all recombinant vectors is performed for the efficient deletion of both hGDH1 and hGDH2 proteins (figure 9).

Materials

- DMEM growth medium (445 ml DMEM Base, 50 ml heat inactivated FBS, 5 ml Penicillin/Streptomycin)
- DMEM growth medium without antibiotics (445 ml DMEM Base, 50 ml heat inactivated FBS)
- Opti-MEM medium
- recombinant plasmid vectors
- lipofectamine 2000

Procedure

The cells are cultured in tissue culture dishes of 60mm size or six wells plates in DMEM growth medium. When the cell culture is 70-90% confluent, the medium is removed and replaced with DMEM growth medium without antibiotics. 4 hours later, for each plate to be transfected is diluted in 125 μl Opti-MEM medium 1 mg recombinant plasmid DNA in a tube. In another tube are diluted 250 μl Opti-MEM with 3 μl lipofectamine 2000. Then, the content of the two tubes is mixed together and the samples are incubated at room temperature for 20 to 25 minutes. After the incubation, the mixture is added dropwise to the cultured cell dish. 4 to 24 hours later, the medium is replaced by fresh DMEM growth medium without antibiotics in order to avoid lipofectamine's toxicity. After 24 to 72 hours the transfected cells are ready for analysis.

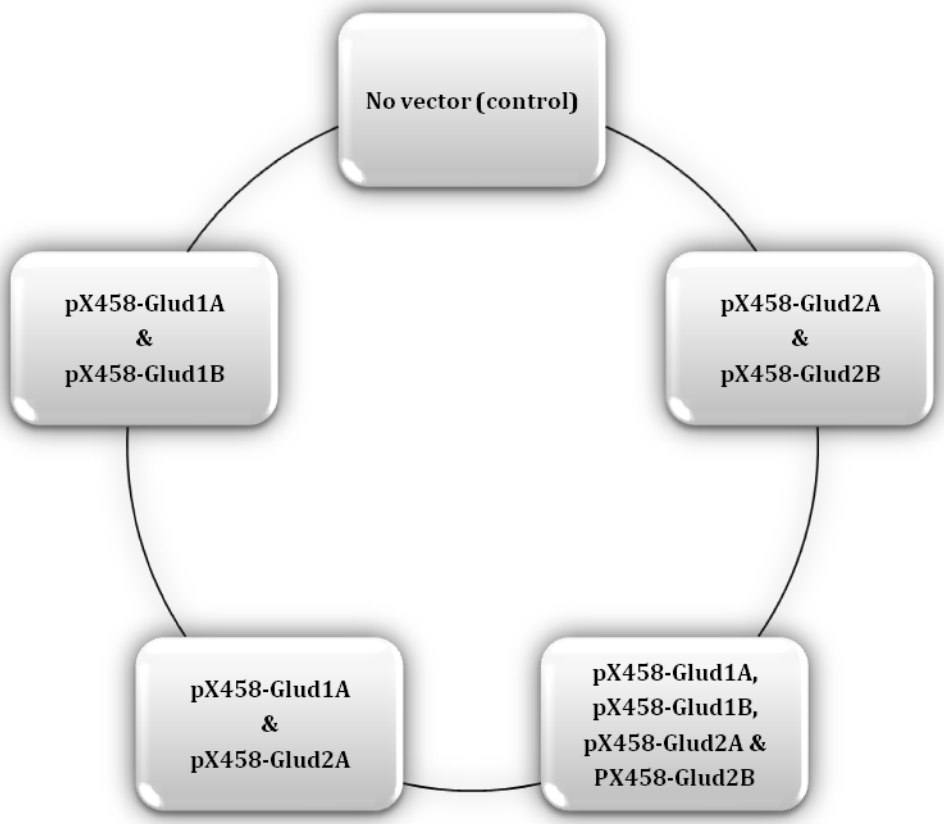

Figure 9: Different combinations of recombinant vectors that are used for the transfection of HEK293 mammalian cell lines. Cells that don't receive any vector are used as a control.

3. Results

3.1. Cloning of the *Glud1*A, *Glud1*B, *Glud2*A, *Glud2*B gRNAs into pSpCas9(BB)-2A-GFP (pX458) plasmid vector

pX458 was selected as the plasmid vector to host our *GLUD1/2* specific gRNAs. Apart from Cas9 gene, which is required for CRISPR/Cas9 gene disruption, the vector also contains an ampicillin resistance gene that permits the selection of the bacterial cells that contain the recombinant vector, and the gene that encodes for the Green fluorescent protein, in order to select the mammalian cell colonies that have successfully received the recombinant vector following their transfection.

Four *GLUD* specific gRNAs were designed. We chose two different gRNAs for each gene in order to increase the knockout efficiency. Moreover, the gRNAs designed for hGDH1 targeting, differed in one or two nucleotides from those that have been designed for hGDH2 targeting so as to ensure discrimination between *GLUD1* and *GLUD2* genes that are highly homologous. First of all, *GLUD* specific gRNAs were annealed to generate double stranded oligonucleotides, and they were subsequently phosphorylated to be capable to form phosphodiester bonds with other DNA chains (through ligation reaction). The vector was digested with BbSI restriction enzyme and was combined with each gRNA. This was possible since the pX458 vector contains two back-to-back recognition sites for BbSI enzyme and after digestion four nucleotides are protruding from each chain. Since the oligonucleotides are designed so as to be complementary with the protruding edges of the vector, ligation procedure is highly efficient (figures 10-13). Nevertheless, after digestion BbSI restriction sites are cut off and are thus lost. The recombinant vectors were introduced into *E.coli* cells via electroporation, and colonies containing the desired insert were selected for further amplification. After that the construct was isolated from the bacterial culture and was sent for sequencing. Sequencing analysis confirmed that each vector contained the desired oligonucleotide (figure 14).

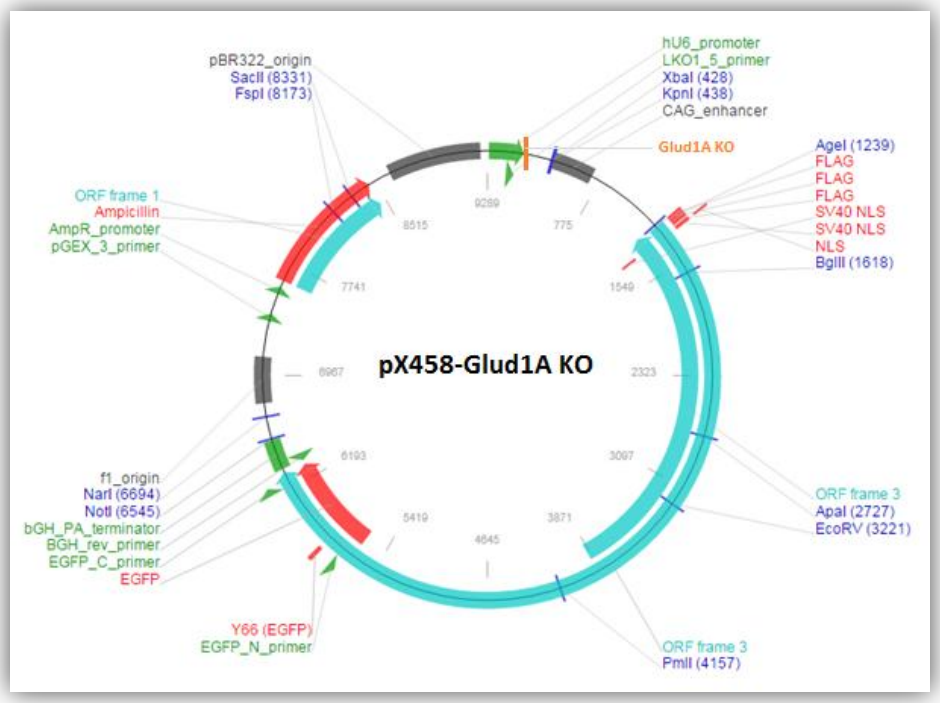

Figure 10: pX458 plasmid vector that contains *Glud1*A KO sgRNA. Most important restriction enzymes that cut pX458 vector are depicted in blue. The one red arrow indicates the ampicillin resistance gene while the other one demonstrates the gene that encodes for GFP. The ORFs are shown with the light blue arrows.

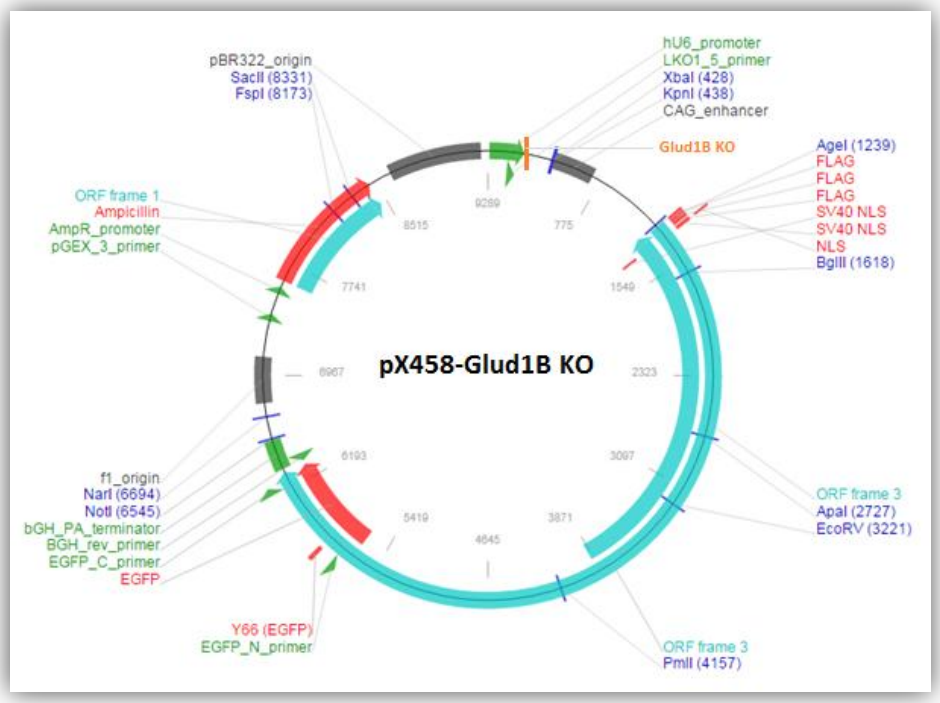

Figure 11: pX458 plasmid vector that contains *Glud1*B KO sgRNA. Most important restriction enzymes that cut pX458 vector are depicted in blue. The one red arrow indicates the ampicillin resistance gene while the other one demonstrates the gene that encodes for GFP. The ORFs are shown with the light blue arrows.

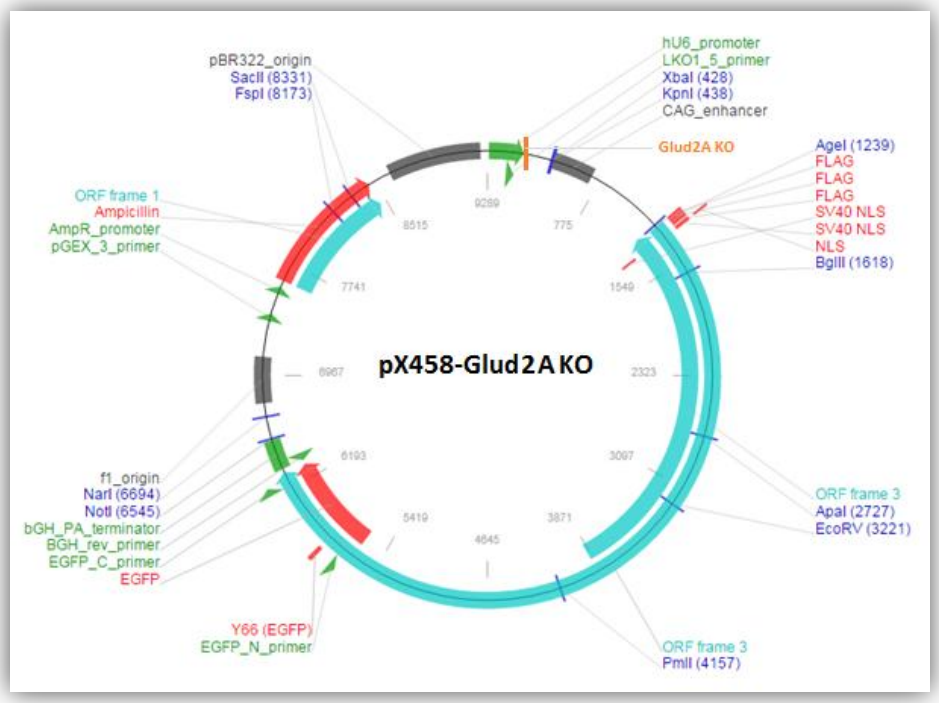

Figure 12: pX458 plasmid vector that contains *Glud2*A KO sgRNA. Most important restriction enzymes that cut pX458 vector are depicted in blue. The one red arrow indicates the ampicillin resistance gene while the other one demonstrates the gene that encodes for GFP. The ORFs are shown with the light blue arrows.

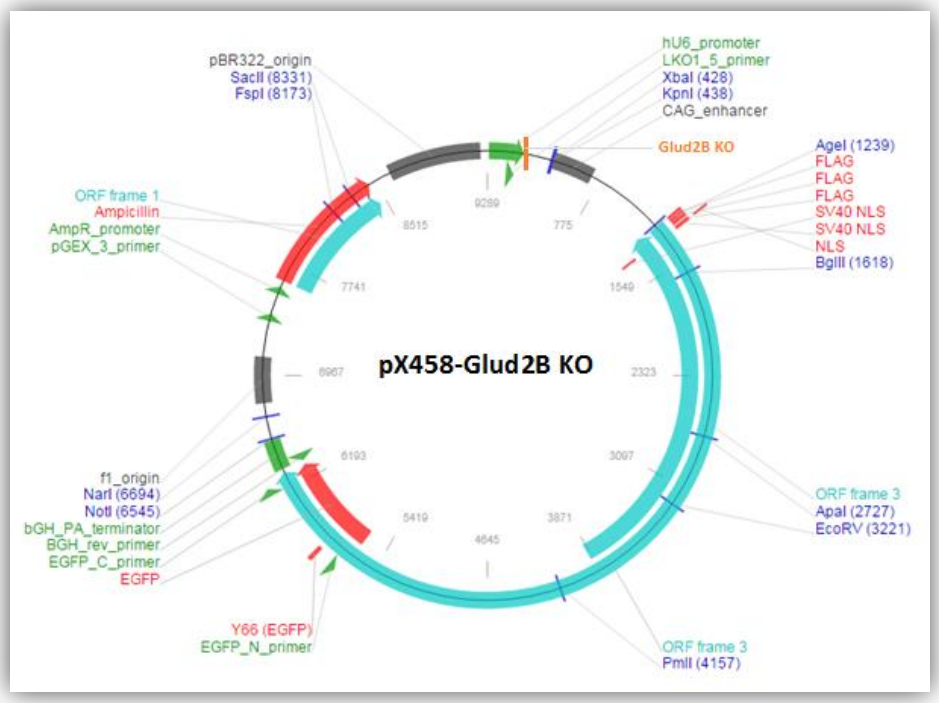

Figure 13: pX458 plasmid vector that contains *Glud2*B KO sgRNA. Most important restriction enzymes that cut pX458 vector are depicted in blue. The one red arrow indicates the ampicillin resistance gene while the other one demonstrates the gene that encodes for GFP. The ORFs are shown with the light blue arrows.

Figure 14: Sequencing analysis of each gRNA cloned into pX458 plasmid vector. In each case, the above line represents the recombinant vector sent to be sequenced and the other line demonstrates the sequence of each designed gRNA. Comparison between these two sequences with the use of "BLAST" online tool revealed that each gRNA has successfully been inserted into pX458 plasmid vector. In addition to this, it was confirmed that no non-specific mutations have been generated throughout the procedure.

3.2. Identification of GDH1 and GDH2 expression in HEK293 wild type cell lysate by Western blotting

Western blot analysis was performed in order to ensure that hGDH1 and hGDH2 proteins can be detected in cell lysates extracted from wild type HEK293 cultures (there have been previous studies in our lab, as well as other studies, that established the presence of both proteins in these cells). Before the loading of the samples on a polyacrylamide gel, a Bradford assay was performed so as to estimate the total protein concentration in the lysate. For the identification of the two isoproteins, two different antibodies, each specific for the respective protein, were used. The intensity of the signal generated from GDH1 was quite low, while GDH2 is rather indistinguishable (figures 15, 16). For this reason, in the future,Western blotting willbe repeated using mitochondrial fractions obtained

from HEK293 cultures, instead of the whole lysate, in addition to optimizing the assay conditions using whole cell extracts. Since GDH is localized in the mitochondrial matrix, we expect it to be more concentrated in that subcellular fraction so that it might be more easily detected by Western Blot.

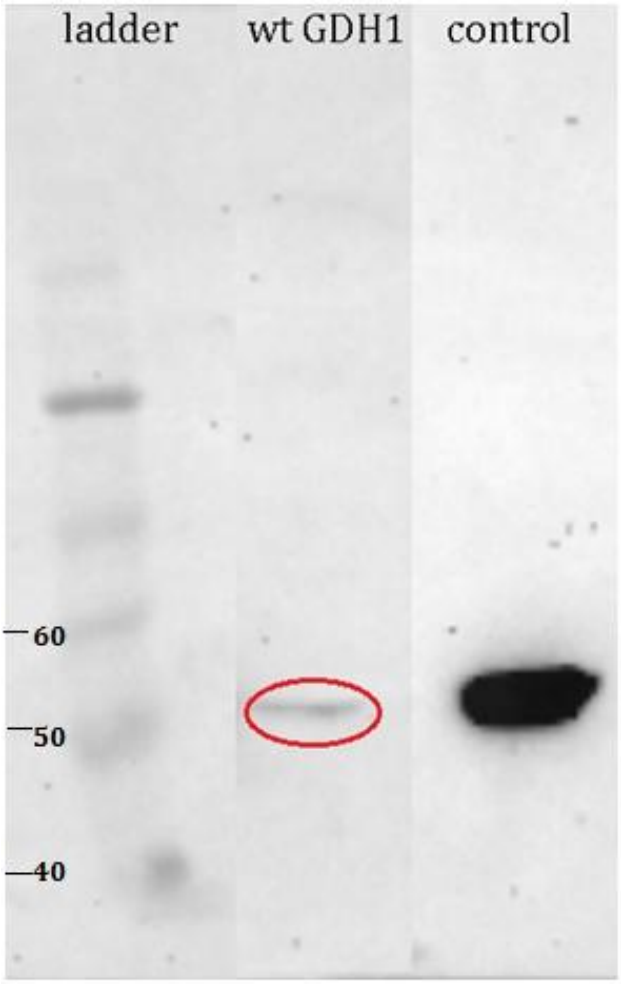

Figure 15: Western blotting analysis for the identification of GDH1. The molecular weight of GDH1 is approximately 56 kDa.

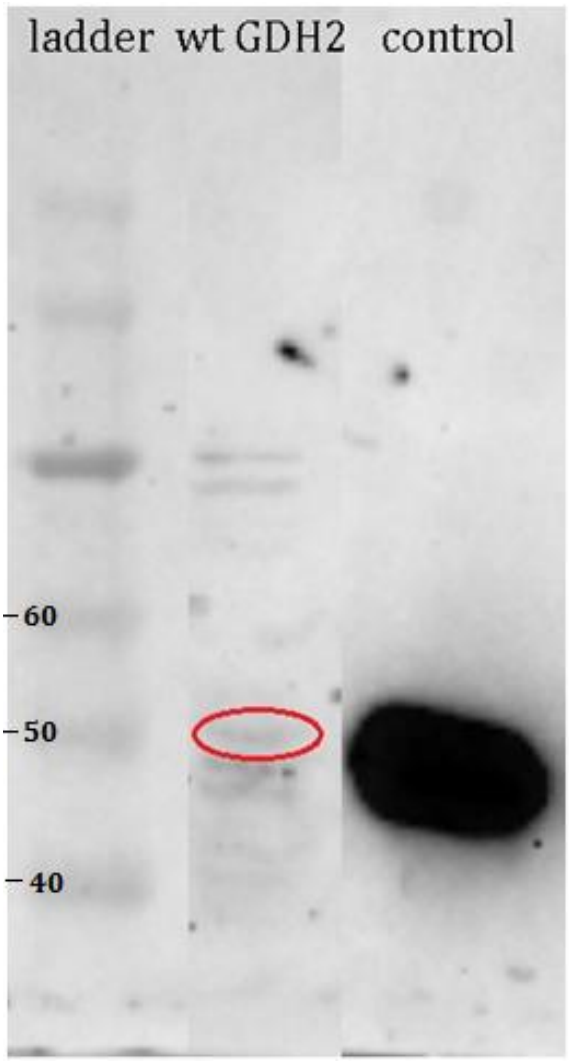

Figure 16: Western blotting analysis for the identification of GDH2. The molecular weight of GDH2 is approximately 56 kDa.

3.3. Transfection of HEK293 cell lines with the recombinant plasmids

The recombinant plasmid vectors are currently used to transfect HEK293 mammalian cell lines in order to disrupt hGDH1 and/or hGDH2 expression (data not shown).

4. Discussion

GDH is an abundant mitochondrial enzyme with a critical role in many cellular functions such as Krebs cycle, insulin secretion and ammonia regulation (Rothe *et al*, 1994). It catalyzes the reversible interconversion of glutamate into a-

ketoglutarate and ammonia, using NADP(H) and NAD(H) as cofactors, and in humans and great apes it is encoded by two different genes, *GLUD1* and *GLUD2*, respectively, that have high homology (Hudson and Daniel, 1993). It has been proposed that *GLUD2* has emerged after retroposition of *GLUD1* from chromosome 10 to chromosome X. This retroposition event is unique since usually a gene of the X chromosome is moved to an autosomal chromosome. This particularity has been proposed to serve the support of some significant events that occur in male spermatogenesis and meiosis (Marques *et al*, 2005). Despite the fact that GDH has been thoroughly studied, only a handful of studies have focused on knockout of *GLUD* genes. More specifically two groups of researchers have attempted to delete GDH1. However, even in these two studies GDH1 was deleted only in specific cell lines/tissues. In addition to this, *GLUD1*-knockout at the level of an organism has never been attempted. Probably such a deletion would be fatal for the organism. The researchers of the first study deleted GDH1 in mice β-cells and observed that part of the insulin secretary response was abrogated. They observed that these mice could still secrete insulin, but in a lower rate. (Carobbio *et al*, 2009). The second group knocked out GDH1 in both directions (oxidative deamination and reductive amination) in the CNS of mice, which resulted in alterations in glutamate handling (Frigerio *et al*, 2012).

This is the first time that hGDH1, which is a ubiquitously expressed protein that has been associated with HI-HA syndrome and other diseases (Stanley *et al*, 1998; Olabarria *et al*, 2011; Kulijewicz-Nawrot *et al*, 2013) will be deleted in kidney (HEK293) cells using CRISPR/Cas system. CRISPR/Cas system that has been chosen for this study is a very promising and innovative technique that has been already successfully used for several knockout studies. In addition to this, GDH2 expression has not been abolished before even though it is a crucial enzyme for many cell functions and has been associated with Parkinson's disease (Plaitakis *et al*, 2010). The effects of hGDH2 deletion on cellular viability are important since *GLUD2* is a newly discovered gene that has not been extensively studied yet. Furthermore, it is important to estimate the consequences of hGDH2 deletion since its birth coincides with brain size increase and functional complexity in the primates (Burki and Kaessmann, 2004).

The current study is the first step for the knockout of these two significant isoproteins in HEK293 cells. The recombinant plasmids that have been constructed in the context of my thesis are currentlyused to transfect mammalian cells. Two of them contain gRNAs specific for the targeting of *GLUD1* and the other two consist of gRNAs designed for the specific deletion of *GLUD2*. Studies are performed on *GLUD1*-single-HEK293-KO, *GLUD2*-single-HEK293-KO as well as *GLUD1/GLUD2*-double-HEK293-KO. After the transfection, DNA will be extracted from HEK293 cells and a polymerase chain reaction (PCR) will be performed with a set of primers binding at either site of our genes, already available in the lab, and will be compared with wild type HEK293 cells. We will also perform sequencing analysis if any PCR product is detected at all, to see how the gene is disrupted. Then, the whole lysate or/and the mitochondria will be extracted and GDH expression will be estimated by Western blotting and compared with the wild type. In addition to this, the mRNA levels of both GDH1 and GDH2 will be estimated using RT-PCR so as to ensure that the knockout of *GLUD* genes has been successfully performed. If cells are viable after transfection, we will choose and isolate single green colonies within two days because after two days, no green colonies will be visible. Then, we will grow them after many split-steps to generate stable cell lines lacking *GLUD1/2* gene(s). Prospectively, cellular viability and mortality rate will be estimated in the GDH deficient cell lines. Furthermore, potential metabolic changes that will be brought about due to the absence of glutamate dehydrogenases will be detected and examined with the use of MTT cell proliferation assay and other techniques (figure 17). Interestingly, in parallel experiments unfolding in our lab, overexpression of hGDH1 and hGDH2 led to increased cellular viability in cultures of stable lines HEK293 in comparison with the wild type cell lines (Mathioudakis *et al*, unpublished data). Ultimately, this study is expected to provide useful insights in the significance of hGDH1/2 for cell viability and metabolic processes.

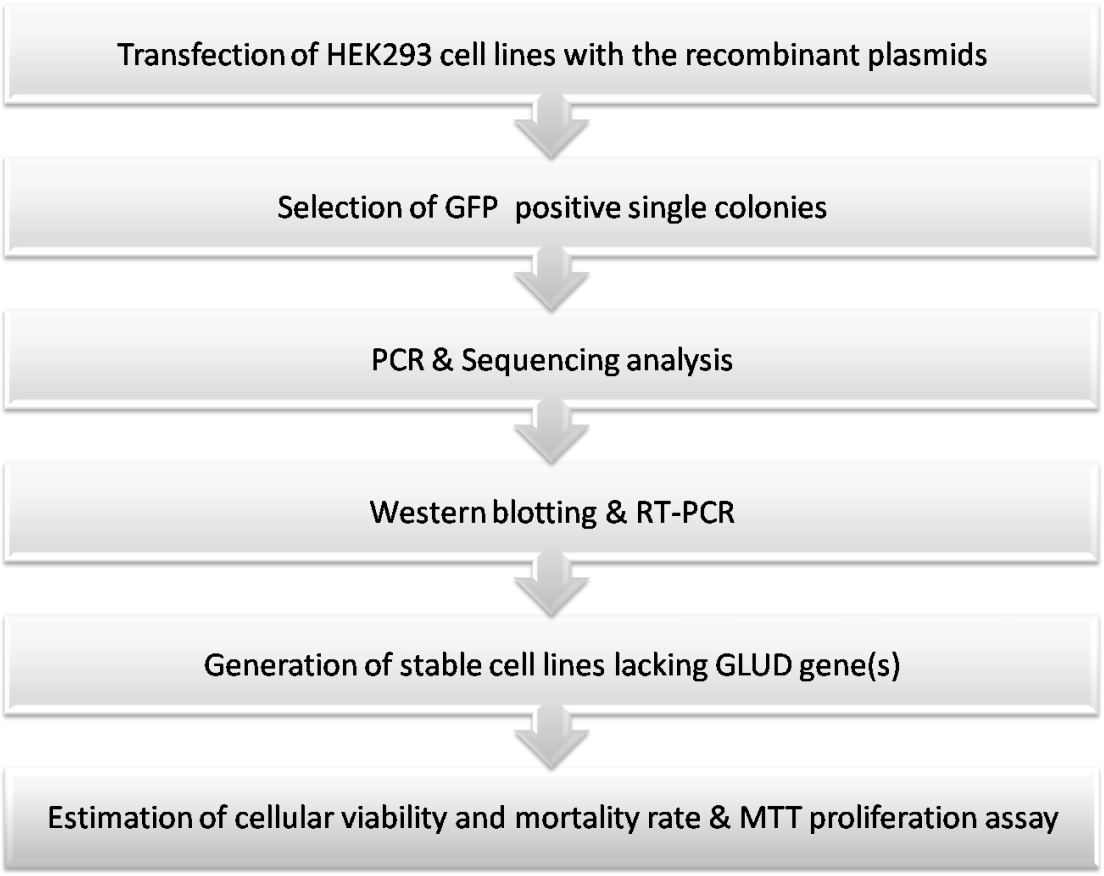

Figure 17: Flowchart of the procedure that will be followed after the transfection of HEK293 mammalian cell lines.

5. References

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