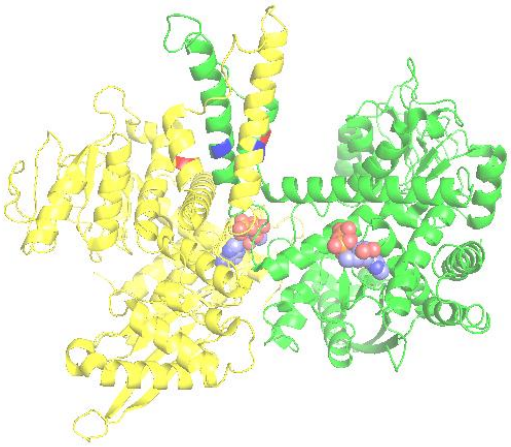
University of Crete
Faculty of Medicine

hGDH1 and 2 over-expression and co-expression in cell lines



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Contents

ΠΕΡΙΛΗΨΗ

Θεωρητικο υπόβαθρο: Η γλουταμική αφυδρογονάση, ένα ένζυμο που υπάρχει σε διάφορους ιστούς θηλαστικών, καταλύει την αναστρέψιμη αντίδραση απαμίνωσης του γλουταμικού σε α-κετογλουταρικό και αμμωνία. Δύο ισομορφές γλουταμικής αφυδρογονάσης απαντώνται στον άνθρωπο, η hGDH1 και η hGDH2, αντιστοίχως. Η hGDH1 είναι ένζυμο που εκφράζεται σε όλα τα κύτταρα του ανθρώπινου οργανισμού. Απεναντίας, η hGDH2 μέχρι στιγμής έχει εντοπιστεί σε κύτταρα του εγκεφάλου, όρχεων και νεφρών. Χαρακτηριστικό των δύο ισο-ενζύμων είναι η αλλοστερική ενεργοποίηση από ADP και λευκίνη καθώς και η αναστολή της δράσης τους από την παρουσία GTP. Οι γλουταμικές αφυδρογονάσες συμβάλλουν σε σημαντικές κυτταρικές λειτουργίες καθώς συνδέουν το μεταβολισμό των υδατανθράκων με αυτόν των αμινοξέων, ρυθμίζουν τον μεταβολισμό του γλουταμικού ως νευροδιαβιβαστή και την έκκρισης ινσουλίνης, και τέλος, συμμετέχουν στην παραγωγή ενέργειας και την ομοιόσταση της αμμωνίας. Προηγούμενες μελέτες έχουν αποδείξει ότι τα ένζυμα αυτά εντοπίζονται στη μήτρα των μιτοχονδρίων.

Στόχοι της μελέτης: Οι στόχοι της μελέτης είναι δύο, ο πρώτος να επιβεβαιωθεί η υποκυτταρική συνεντόπιση των hGDH1 και hGDH2 και ο δεύτερος να μελετηθεί το αποτέλεσμα της υπερέκφρασης hGDH1 και hGDH2 σε κυτταρικές σειρές.

Μέθοδοι: Ως προς τον πρώτο στόχο της παρούσας μελέτης, έγινε ιχνηθέτηση των ενζύμων με διαφορετικών χρωμάτων φθορίζουσες πρωτεΐνες, ώστε να επιβεβαιωθεί η υποκυτταρική τους συνεντόπιση. Ως προς τον δεύτερο στόχο της μελέτης, έγινε δημιουργία κυτταρικών σειρών HEK293 που υπερεκφράζουν είτε hGDH1 είτε hGDH2, ώστε στη συνέχεια να μελετηθεί ο αντίστοιχος φαινότυπος.

Αποτελέσματα: Τα πειραματικά αποτελέσματα των μελετών μας σχετικά με τον εντοπισμό των δύο ανθρώπινων γλουταμικών αφυδρογονασών υποδηλώνουν ότι τα ένζυμα αυτά συνεντοπίζονται υποκυτταρικά εντός των μιτοχονδρίων. Συγκεκριμένα, έπειτα από ιχνηθέτηση της hGDH1 με πράσινο χρώμα και της hGDH2 με κόκκινη φθορίζουσα πρωτεΐνη, επιβεβαιώνεται σύμφωνα με τη διακριτική ικανότητα του μικροσκοπίου που χρησιμοποιήθηκε ότι συνεντοπίζονται στα μιτοχόνδρια. Επίσης, σύμφωνα με τα πειράματα που πραγματοποιήθηκαν για το φαινοτυπικό χαρακτηρισμό των κυτταρικών σειρών που δημιουργήθηκαν, τα αποτελέσματά μας υποδηλώνουν ότι τα κύτταρα HEK293 που υπερεκφράζουν είτε hGDH1 είτε hGDH2 εμφανίζουν μειωμένα ποσοστά νεκρών κυττάρων σε διάφορα στάδια της ανάπτυξης τους σε καλλιέργεια. Συγκεκριμένα, το ποσοστό νεκρών κυττάρων που αναγνωρίστηκαν στην καλλιέργεια 24 ώρες μετά την σπορά των κυττάρων, ήταν περίπου 35% στα αγρίου τύπου κύτταρα, ενώ στις κυτταρικές σειρές που

υπερεκφράζουν hGDH1 και hGDH2 ήταν 10-15% (p value<0,0001) και 5–15% (p value<0,0001), αντιστοίχως.

Συμπέρασματα: Συμπερασματικά, τα πειραματικά μας αποτελέσματα δείχνουν υποκυτταρική συνεντόπιση hGDH1 και hGDH2, και σημαντικό ρόλο και των δύο στην κυτταρική ανάπτυξη. Τα αποτελέσματα αυτά χρειάζεται να αναλυθούν και να διερευνηθούν εκτενέστερα, ώστε να εξακριβωθεί ο ρόλος των ανθρώπινων ισοενζύμων γλουταμικής αφυδρογονάσης στην κυτταρική λειτουργία και βιωσιμότητα.

ABSTRACT

Background: Glutamate dehydrogenases catalyze the reversible deamination of L-glutamate to a-ketoglutarate and ammonia. Two isoforms of glutamate dehydrogenases are found in humans, namely hGDH1 and hGDH2. hGDH1 is expressed in almost every human cell (housekeeping enzyme). On the other hand, hGDH2 has been found mainly in brain, testis and kidneys. Both enzymes are allosterically activated by ADP and L-leucine and inhibited by GTP. They contribute to important cellular processes as they interconnect amino acid and carbohydrate metabolism, mediate neurotransmitter recycling and play important role in energy production, ammonia management and insulin secretion. Previous studies have indirectly proven that both enzymes are located in mitochondria matrix.

Aim of the study: The present study is divided in two main complementary objectives. The first objective is to determine whether the two isoenzymes are co-localized subcellularly. The second objective is to study the result of the over expression of hGDH1 and hGDH2 in mammalian cell lines.

Methods: For answering our first scientific question, we tagged each iso-enzyme with fluorescent protein of different colour, so that we could detect them subcellularly using confocal microscopy. For the second objective, we created stable HEK293 cell lines over expressing either hGDH1 or hGDH2 in order to study their phenotypic characteristics.

Results: Experiments related to subcellular localization, revealed that the two enzymes are co-localized inside mitochondrial matrix. Specifically, after tagging hGDH1 with GFP and hGDH2 with RFP, it was confirmed, as far as the resolution of our confocal microscope permitted, that they co-localize inside the mitochondrial matrix. Moreover, characterization experiments showed that stable HEK293 lines over expressing hGDH1 or hGDH2, respectively, display lower death rates in different time points of their lifespan as compared to wild-type cells. Specifically, mainly in exponential growth phase (24h after plating), WT HEK293 exhibit more dead cells (approximately 35%) compared to stable cell lines over expressing hGDH1 and hGDH2 (10-15%; $p<0.0001$ and 5-15%; $p<0.0001$, respectively).

Conclusions: In conclusion, our experimental results suggest subcellular co-localization of hGDH1 and hGDH2, as well as major contribution of both iso-enzymes in cell growth and lifespan. These data need to be further analyzed and followed by additional studies for deciphering the role of hGDHs in cell viability and function.

1. INTRODUCTION

Human brain constitutes approximately 2% of total body weight. Nevertheless, it receives 10% of cardiac blood supply output and consumes 25% of total glucose and 20% of total blood oxygen during cerebral activity (Magistretti P. J., 1999). Glucose is the main energy substrate for the brain. It can be processed through various metabolic pathways, although in neural tissue glucose is fully oxidized into CO_2 and water through the processes of glycolysis, tricarboxylic acid cycle for aerobic metabolism and associated oxidative phosphorylation. This process yields 30 to 38 molecules of ATP for each molecule of glucose oxidized and is very effective at meeting the energy demands of working cells.

Cellular metabolism comprises two major groups of reactions, anabolism and catabolism. Anabolism is the use of energy in order to synthesize the necessary structural components of the cells, such as nucleic acids and proteins. With the term catabolism we refer to the reactions used for breaking down organic matter in order to produce energy. This procedure is called cellular respiration.

Glucose is the main fuel used for cellular respiration and is used by the cells through glycolysis for the production of ATP. Through glycolysis, one molecule of glycose is finally metabolized into two molecules of pyruvate. These reactions are taking place without the presence of oxygen and as final outcome they produce two molecules of ATP (Fig 1.1).

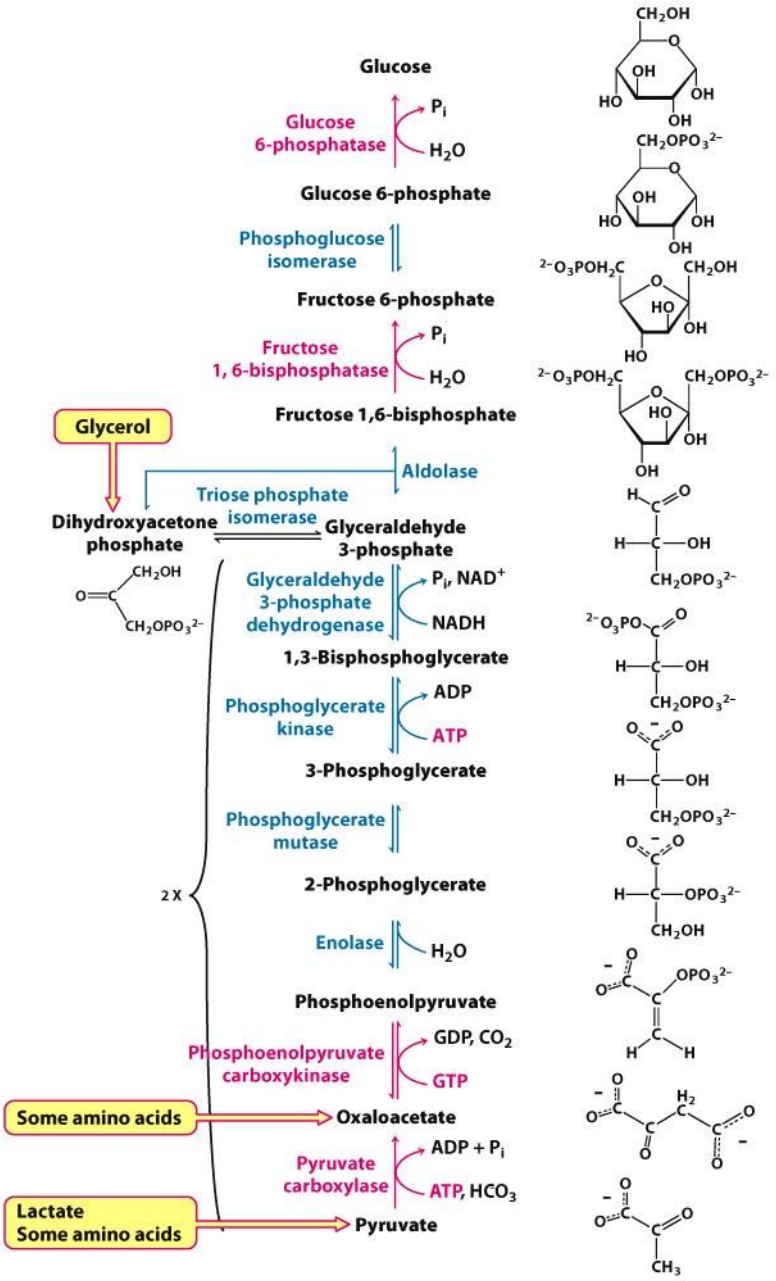

Figure 16.24
***Biochemistry,* Seventh Edition**

Fig. 1.1 Schematic representation of glycolysis pathway (Kevin Ahern, Biochemistry).

Moreover, the production of pyruvate is not the end of cellular metabolism of glucose. On the contrary, pyruvate is transported to the mitochondrial matrix, were there exist enzymes that degrade it for additional energy production. This time, the procedure requires the presence of oxygen. It is from that point on, that mitochondria take action. Another advantage of further degradation, besides the energy production, is the reduction of pyruvate in the cell, as well as the regulation

of levels of lactate. Lactate is synthesized and accumulates under intense muscular exercise and can become toxic for the cells in higher concentrations. Pyruvate is small enough for entering into mitochondria as a fuel for another cascade of chain reactions. That series of reactions, comprise the tricarboxylic acid cycle (TCA) or Krebs cycle, (Fig. 1.2) and happen inside the mitochondrial matrix resulting in the production of energy with the use of oxygen. During the Krebs cycle, ATP molecules are produced as well as 3 NAPH and 1 $FADH_2$. NADH and $FADH_2$ are transferring H^+ to the respiratory chain resulting in more energy production. If we calculate the total amount of ATP produced in each full reaction cascade it is around 20 molecules. That way the cells can ensure the optimal amount of energy from nutrition, to cover even the most demanding, energy spending cells in the organism.

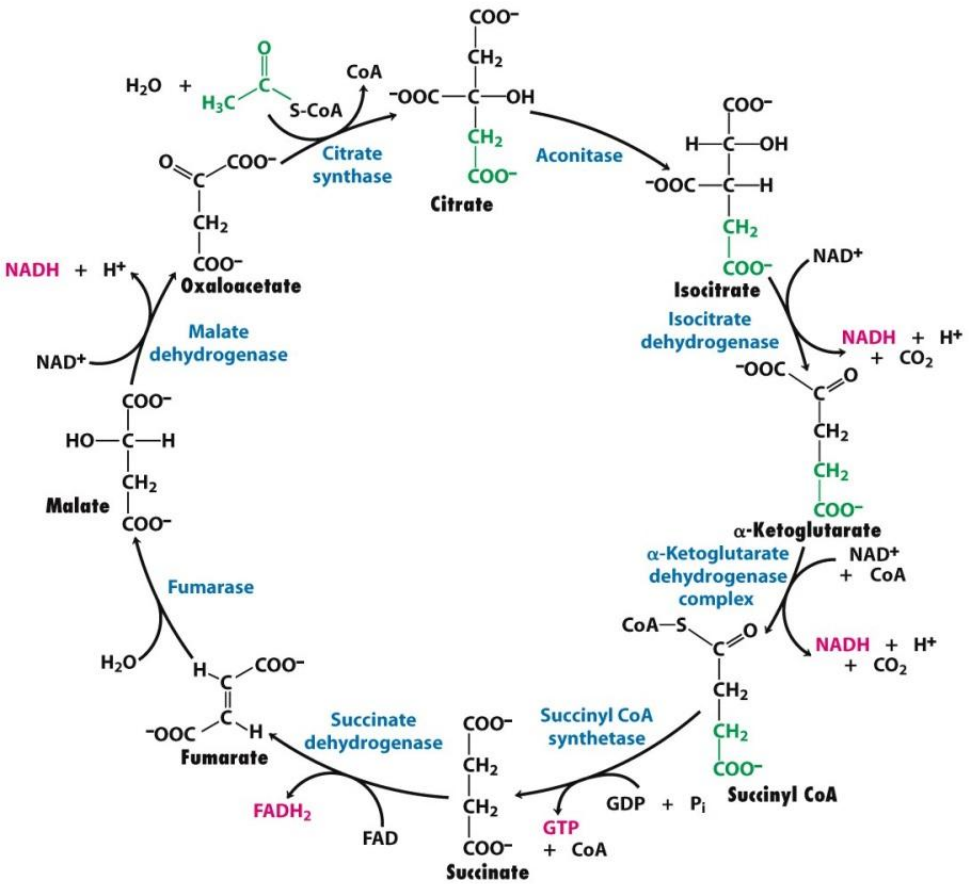

Figure 17.15
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Fig. 1.2 Schematic representation of tricarboxylic acid cycle (TCA) or Krebs cycle (Kevin Ahern, Biochemistry)

GLUTAMATE DEHYDROGENASE (GDH)

Glutamate dehydrogenase is an enzyme which fuels aerobic metabolism through Krebs cycle inside mitochondria. It is a key enzyme for metabolism and consequently cell proliferation, thus it can be found in most of living species (Hudson RC and Daniel RM, 1993). In humans two isoforms can be found, hGDH1 and hGDH2 (Shashidharan P. et al., 1994). Glutamate dehydrogenase catalyzes the reversible deamination of L-glutamate to a-ketoglutarate (an intermediate of Krebs cycle) and ammonia (Stryer L. Biochemistry volume I) In order to perform this reaction, hGDH uses NAD+ and NADP+ as cofactors. It contributes to important cellular processes as it interconnects amino acid and carbohydrate metabolism, mediates neurotransmitter recycling, and plays important roles in energy production, ammonia management and insulin secretion (Plaitakis A. et al, 2013). Mutations that result in activation of this enzyme are a common cause of congenital hyperinsulinism. This enzyme is allosterically activated by ADP, leucine and inhibited by GTP and ATP. (Fig. 1.3)

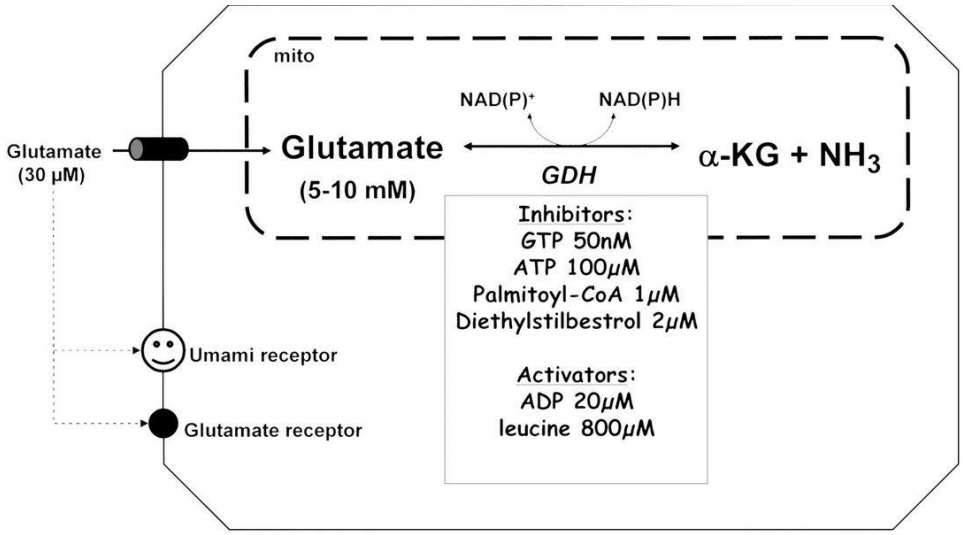

Fig. 1.3 Glutamate metabolism and insulin secretion. The diagram illustrates the reversible deamination reaction of hGDHs. A list of the enzyme activators and inhibitors are also provided (Stanley C.A., 2009)

Even though that reaction is reversible, there seems to be a preference in humans to the direction of the production of a-ketoglutarate. One important parameter, except of energy production, is the regulation of ammonia homeostasis

(Adeva M. et al. 2012). Although according to the reaction's direction, the production of ammonia inside the cells is favored, we must not forget that we are dealing with a reversible reaction which in the inverse direction it catalyzes ammonia fixation. In order to predict the reaction direction at each time point of cellular metabolism, it is wise to keep in mind that many factors could influence the outcome. A good example is a research paper published in 2013 from Zaganas et al. showing the impact of PH inside cell's cytosol and/or mitochondria on reaction's direction and consequently on ammonia's homeostasis. On the other hand, according to Cooper et al, 2012 the major player in ammonia detoxification seems to be glutamine synthetase (GS) and not GDH, an enzyme which contributes to glutamine synthesis from available glutamate in the cells with the use of ammonia. From its mechanism of action to the regulation of ammonia homeostasis in the human tissues, it becomes evident that the glutamate dehydrogenases play a key role as reviewed from Spanaki C. and Plaitakis A., on 2012.

STRUCTURE OF GLUTAMATE DEHYDROGENASES

Glutamate dehydrogenases are proteins that can be found among most of living species due to their importance in energy production. But according to the different energy consumption and demands among species they need to adopt and evolve. It is maybe for that reason, that if we search between species we can find many minor differences in enzyme structure, even many isoforms that serve the same function in the cells. In some of the higher primate species, such as chimpanzee, gorilla, orangutan, gibbon and human it has been found that there are two genes expressing GDH, namely GDH1 and 2. GDH1 is a housekeeping protein, found in all the mammals, while GDH2 is found only in the primates mentioned above (Burki F. & Kaessmann H., 2004). In human DNA the two "functional" genes coding GDH are *GLUD1* and *GLUD2* and are located in different sites of the human genome.

The housekeeping gene *GLUD1* is expressed in all human tissues and located in chromosomal location 10q23.3. The size of the gene is 44818 base pairs long. It contains 13 exons and 12 introns. Exons correspond to 1677 of base pairs, coding for a peptide of 558 amino acids which is the final size of the enzyme produced.

On the other hand, *GLUD2* gene located in chromosome X is expressed in specific human tissues such as brain, testis and kidneys (Spanaki C. et al., 2014). That gene contains no introns and for that reason it is believed that *GLUD1* mRNA was reversely inserted in chromosome X. That retroposition was possibly amended by natural selection and random mutations created the *GLUD2* gene. That gene is 2335 base pairs long. If we carefully revise the coding parts of both genes (*GLUD1* and *GLUD2*) we will ascertain that they represent 96% homology (Shashidharan P. et al., 1994). *GLUD2* gene also leads to a polypeptide 558 amino acids long (Gdh2) with few differences from GDH1. The human *GLUD1* gene sequence and the differences it has from *GLUD2*, are represented on the picture below. Also the same information at the translation level is highlighted. (Fig.1.4)

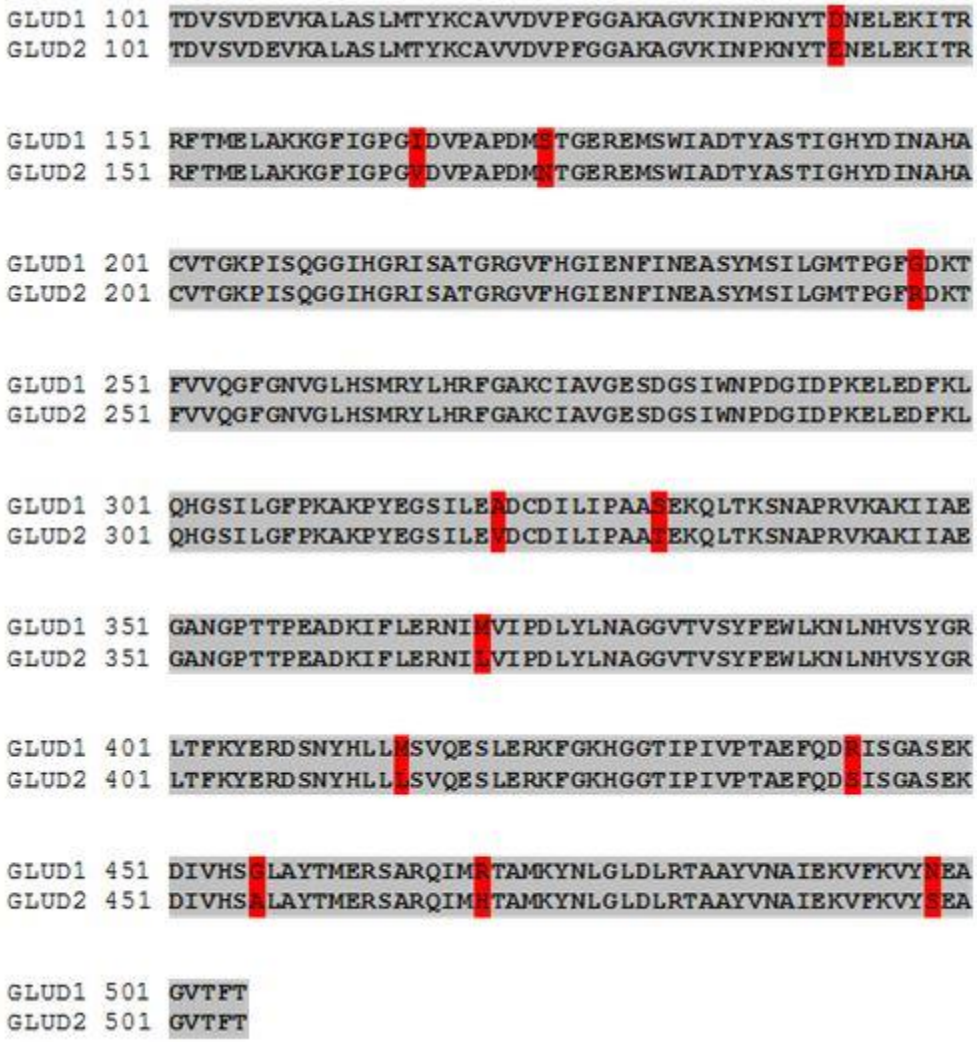

Fig. 1.4 : Comparison between the sequence of *GLUD1* and *GLUD2* human genes. The differences between the two enzymes are highlighted in red (Shashidharan P. et al., 1994, modified)

As observed above, glutamate dehydrogenases 1 and 2 have generally similar size and structure, except of changes in 15 amino acids that represent 4% of their total length. Even though they are so similar they seem to exhibit differences in functional properties such as thermal stability, basal activity and allosteric regulation properties (Kanavouras K. et al., 2007)

The first 53 amino acids of each protein represent the leader peptide, a region that systematically leads the enzyme in the mitochondrial matrix. At the mitochondrial membrane, the leader peptide is dissected and the mature enzyme of 505 amino acids is finally entering inside mitochondria matrix. That mature enzyme later on forms the subunits of "ready for action" hexamers.

Even though, the two isoenzymes differ in the leader peptide in 9 out of 53 amino acids they seem to both have the same subcellural localization. That is the mitochondrial matrix. It is clear that the leader peptide is responsible for that localization due to results that represent that its removal does not lead the enzyme inside the mitochondria matrix but to the cell cytosol (Mastorodemos V. et al., 2009). Studies are on the way for establishing whether the whole or a/what part of that leader peptide is crucial for that function (Kotzamani D. unpublished data). The detection of GDHs inside mitochondrial matrix is something expected, because it is there that the aerobic metabolism of Krebs cycle is taking place.

It was previously reviewed that the mature enzyme in humans consists of 505 amino acids providing an enzyme of molecular weight of ~56kDa, which formulates hexamers probably for improving functional capacity. It is important to review the final folding form that this polypeptide chain is taking. That information was provided by affinity crystallography. By using this technique, many "snapshots" of the structure of the mammalian GDH was achieved. The protein hexamer crystallized originated from bovine but it is generally known to be very close to human GDH structure (Li M. et al., 2012, 2014). The crystallized form of human GDH is represented below (Fig. 1.5) (Smith TJ. et al., 2002).

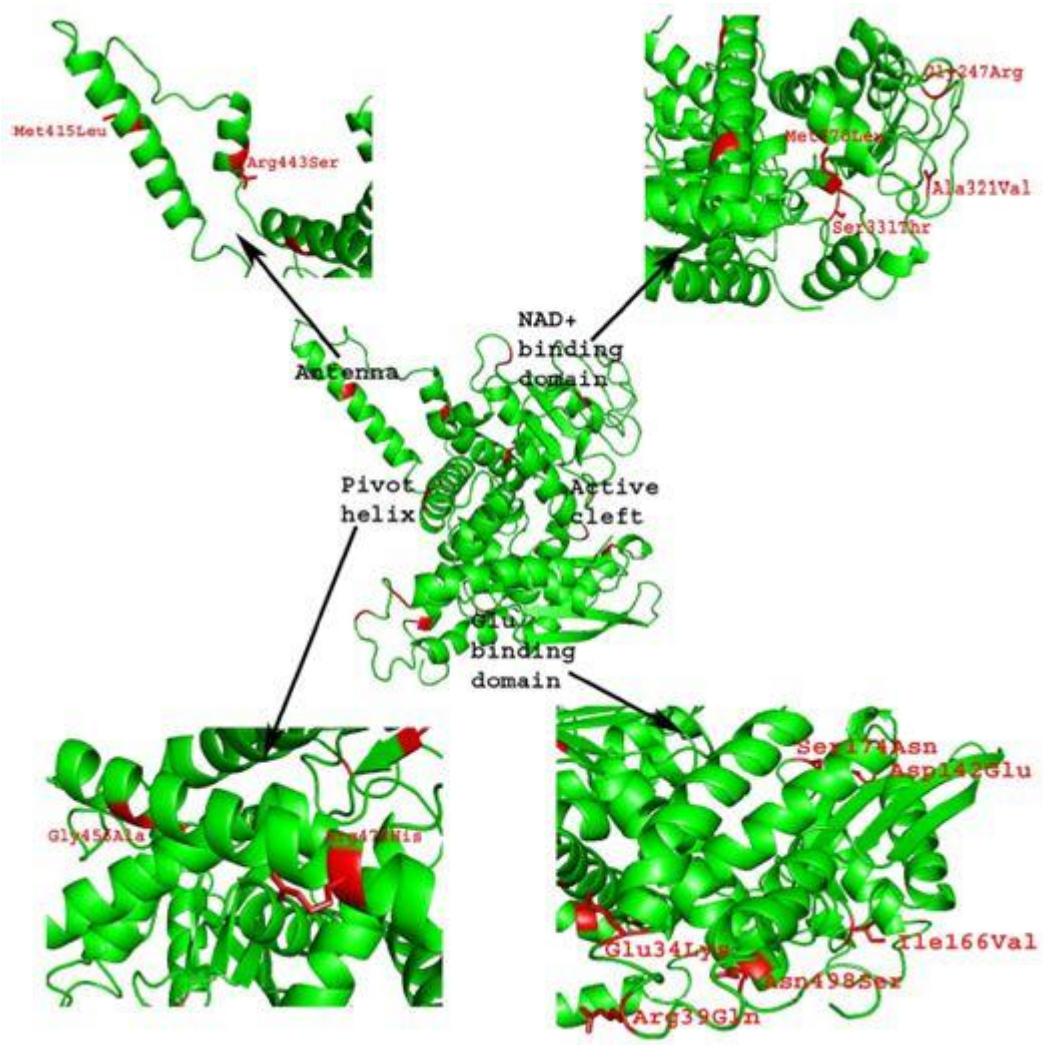

Fig. 1.5 The crystallized 3D representation of human glutamate dehydrogenase 1 as achieved from Smith et al., 2002 (Zaganas I. et al., 2014).

In the final form of GDH, we can discriminate some structural characteristics that provide the enzyme with the ability to have specific regulatory function. The parts of the enzyme that seem to be particularly important are the NAD+ binding domain, the glutamate binding domain, the pivot helix, the active cleft and the antenna (illustrated in the picture above). In the figure above, differences between GDH2 and GDH1 are depicted in red color, although a crystallography structure of GDH2 has not been published yet (Zaganas I. et al., 2014).

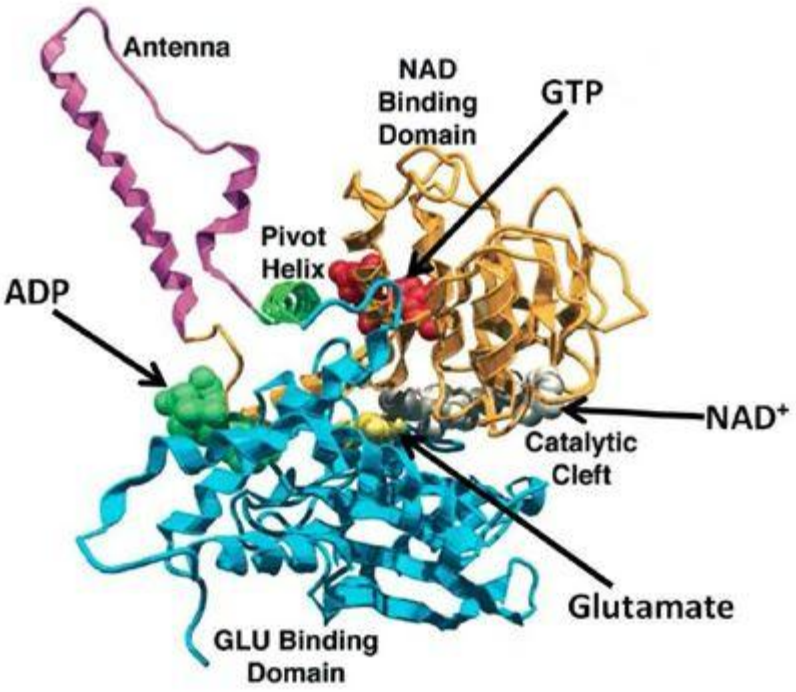

Fig. 1.6 The structure of mature human GDH1 as achieved through affinity crystallography, with the binding sites for GTP, ADP, glutamate and NAD+ (Allen A. et al, 2004, Kanavouras K. PhD thesis, 2012)

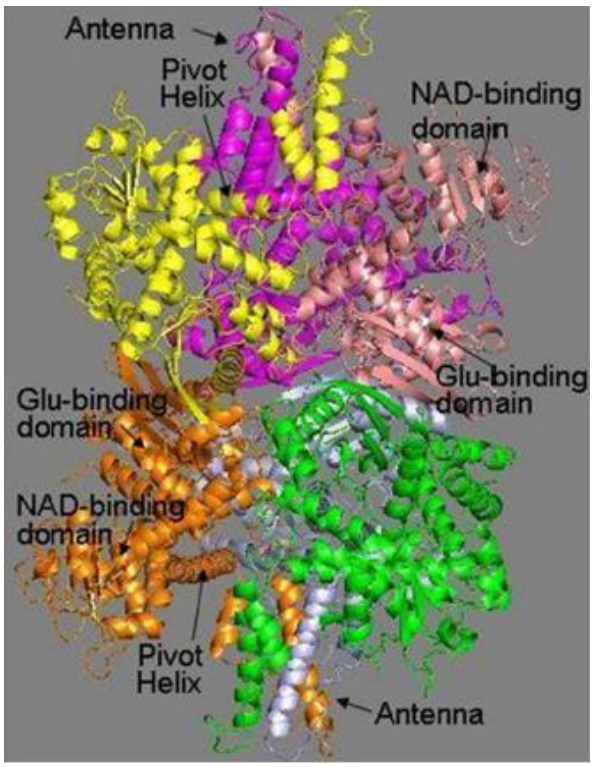

Fig. 1.7 The hexameric form of hGDH1. Each subunit is represented with a different colour

In the figure 1.7 above, it is represented the hexamer of the GDH1. There is a different color representation for each subunit of the hexamer. It is believed that this is the final functional form of the enzyme inside mitochondrial matrix (Smith et al., 2002, Zaganas I. et al., 2014).

FUNCTIONAL DIFFERENCES BETWEEN GDH1 AND GDH2

In the previous section, the structure of the two isoforms of glutamate dehydrogenase in humans was reviewed. The two enzymes are very similar in most of their structure characteristics in molecular and protein level (~96%). In this section it will be shortly reviewed if they represent any differences on their functional characteristics.

Experiments, measuring hGDH1 and hGDH2 activity and function, highlighted many different responses of these isoenzymes. Data from enzymatic activity measurements provide information about their enzymatic inhibition by GTP and their resistance to thermal inactivation. In order to be more specific, even though we know that GTP inhibits both enzymes, it seems that they have a different sensitivity to that inhibition. hGDH1 seems to react strongly in GTP inhibition, while hGDH2 shows a marked resistance to inhibition from this compound, regardless the presence or absence of ADP. According to the matter of thermal inactivation, it seems that hGDH2 is far more sensitive than hGDH1 exposed in 45°C (Kanavouras K. et al., 2007). The substitution of Gly456Ala that might be evolutionary formulated in the gene of *GLUD2*, seems to be responsible for the loss of sensitivity in cooperative binding of hGDH2 to GTP (Zaganas and Plaitakis, 2002).

Another difference between these enzymes seems to be on basal activity, as hGDH2 shows 3%-8% basal activity and hGDH1 represents ~35% in the absence of activators. But hGDH2 is fully responsive to ADP or L-leucine and those activators are capable of fully restoring and equalizing the Vmax of both enzymes. ADP shows a lower affinity for hGDH2 than hGDH1, but L-leucine activates both enzymes with equal affinity (Zaganas et al., 2002, Plaitakis A. et al., 2011).

Their maximal specific activity seems to differ under alterations in pH. It seems that they have different optimal pH for providing their maximum specific activity. The optimal pHfor hGDH1 is 7,75-8,0, and 7,50 for hGDH2 (Kanavouras K. et al., 2007). That performance of hGDH2, is thought to be achieved evolutionary

because of the adaptation in the acidic environment of neural astrocytes after excitatory neurotransmission and synaptic glutamate uptake (Plaitakis A. et al., 2011).

EXPRESSION OF HUMAN GLUTAMATE DEHYDROGENASES IN NEURONAL AND NON NEURONAL TISSUES

Another major difference between GDH1 and GDH2 is the pattern of expression among human tissue. As we already mentioned, hGDH1 is a housekeeping enzyme, which means that it is expressed in all human tissues. On the other hand hGDH2 has a specific pattern of expression in human tissues. It is expressed in human brain lobes (frontal, parietal, temporal), in testis (sertoli cells) and kidneys according to the bibliography so far. hGDH1 is expressed in the same tissues but in bigger amounts than hGDH2. Immunohistochemical studies revealed that in human brain tissue it is expressed more intensely in astrocytes than in neurons. (Zaganas I. et al., 2012, Spanaki C. et al., 2010).

Of note, astrocytes are the supportive cells of the brain, much bigger than neurons and their main role is to provide neurons with nutrients or chemical compounds necessary for their smooth and optimal function. More specifically they tend to remove the abundant glutamate from the synaptic cleft for avoiding the major side effects from the action of glutamate such as excitotoxicity. The glutamate that is reuptaken by astrocytes from the synaptic cleft, is transformed in a more stable form of glutamine using the enzyme, glutamine synthetase. Astrocytes also produce plenty of glutamate, that way they can provide neurons with glutamine to transform it back to glutamate using neuronal phosphate-activated glutaminase when needed for energy demands or to use as neurotransmitter. This way neurons can use astrocytes capability of glutamate – glutamine transformation and this highly attributes to their TCA cycle (Zaganas I. et al., 2012).

SUBCELLULAR LOCALIZATION OF hGDHs

In the literature there are two main studies dealing with the matter of subcellular localization of the hGDH1 and hGDH2. Both studies have concluded that the enzymes are mainly localized inside the mitochondrial matrix (Mastorodemos V. et al., 2009, Rosso L. et al., 2008). In order to interpret that goal, they constructed a

plasmid vector chimerically expressing hGDH1 or HGDH2 with a GFP. By using that construct they could detect the enzymes localization subcellularly. In both of these studies the results were visualized by using either confocal microscopy or western blot analysis. Both techniques revealed strong signal coming from the enzymes located in mitochondria. This was further confirmed by using another plasmid vector (mito tracker) which stains mitochondria with an RFP.

POSSIBLE INVOLVEMENT OF GLUTAMATE DEHYDROGENASES IN THE PATHOPHYSIOLOGY OF HUMAN DISEASES

HYPERINSULINISM - HYPERAMMONEMIA SYNDROME

Mutations that lead to complete "loss of function" of hGDHs haven't been reported, maybe because of the crucial role of that these enzymes play in the organism that such mutations could result to death. Hyperinsulinism-hyperammonemia (HI/HA) is a syndrome that is proven to be caused by a gain of function mutation in GLUD1 gene. The main clinical manifestations of this syndrome are the hypoglycemia and epileptic seizures that are taking place usually after a rich in protein, meal. People that suffer from HI/HA syndrome usually experience epileptic seizures and have 2-5 times more blood ammonia levels that seem to be asymptomatic.

The mutations that have been reported to cause HI/HA syndrome are those that eliminate the sensitivity of GTP inhibition on the enzyme. In other words, this means that the β-pancreatic cells, that are responsible for the insulin secretion, produce more insulin because of the hGDH hyperactivity. That happens because, under normal conditions, β-pancreatic cells sense ATP/ADP ratio for regulating the insulin secretion. GDH can't stop producing ATP because of low affinity of inhibition by GTP. Redundant ATP in the cells can cause specific ATP sensitive potassium channels to close. As a result, reduced depolarization of the membrane is taking place, after the activation from insertion of calcium, that lead to continuous secretion of insulin (Stanley C.A., 2009)

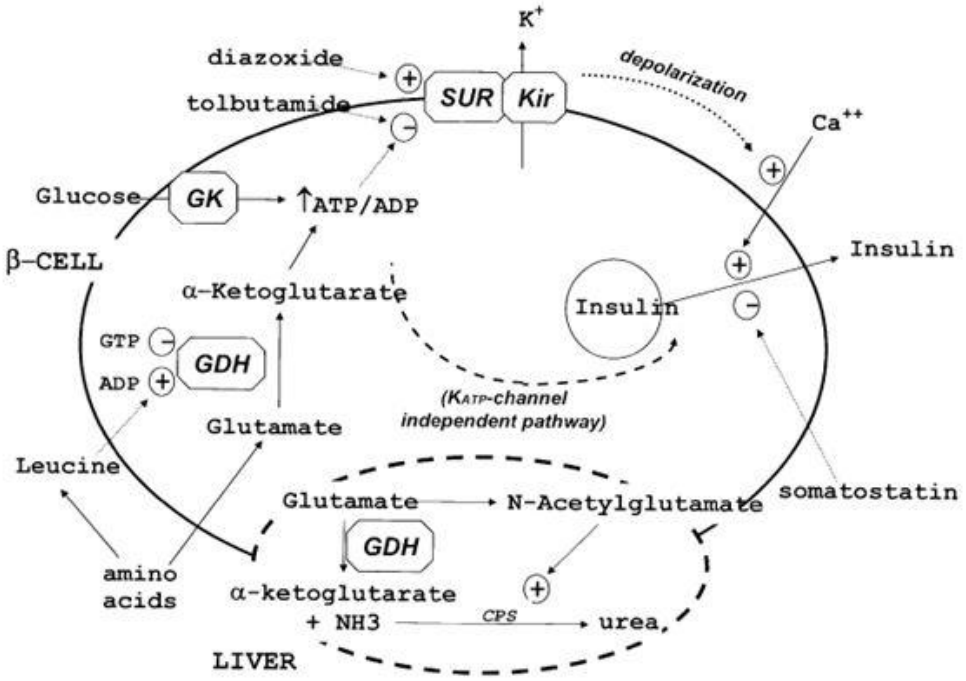

Fig 1.8 : The cascade of reactions resulting HI/HA in pancreatic cells (Stanley C.A., 2009)

hGDH2 AND PARKINSON'S DISEASE

Parkinson's disease exhibits multifactorial etiology. Malfunction in mitochondria has been reported over the years that could result in the onset of the disease. In a way, an unexpected result was published from Plaitakis A. et al., on 2010, revealing that a mutation on human GLUD2 gene has impact on Parkinson's disease onset. In this study they provide evidence that a rare T1492G variant which substitutes Ala for Ser445 in hGDH2 , can accelerate disease onset for 6 – 13 years in men. That mutation in hGDH2, causes a gain of function in the enzyme's basal activity as it seems to represent reduced sensitivity on GTP inhibition, but also high sensitivity on suppressing modification by estrogens. That hypothesis is gaining ground according to the results of a study showing that female heterozygous PD patients have low effect on the disease's onset and this could be due to the suppressive results of estrogens on hGDH2 (Plaitakis A. et al., 2010, 2011). We can only hypothesize about Ala445-hGDH2 variation on human brain but, it is likely that augmentation of glutamate oxidation could accelerate an ongoing degenerative process in PD by altering the compartmented metabolism of glutamate in brain and/or by increasing ROS production (Plaitakis A. et al., 2010). The impact that

hGDH2 has in the pathophysiology of Parkinson's disease remains elusive, although it is for sure a finding that attracts a lot of interest for future research.

OTHER DIRECT OR INDIRECT EVIDENCE OF HGDH INVOLVEMENT IN NEUROLOGIC DISORDERS

There are several studies in the bibliography that connect the action of hGDH with various neurologic conditions. One of the main clinical manifestations that accompany patients with HI/HA syndrome is epilepsy, as already mentioned. At this point, maybe it is wise to remind that one of the main causes of HI/HA syndrome is gain of function mutations in GLUD gene. In the cases that the cause of the syndrome is the hyperactivity of hGDH, it is believed that the mutations in enzyme alone could lead to the development of epilepsy. This is believed because patients that are treated for HI with diazoxide, do not necessarilly stop suffering from epileptic seizures. The mechanism of action of the enzymes can explain that, since according to the authors the hyperactivity of GDH can lead to depletion of glutamate and consequently depletion of neurotransmitter GABA with is synthesized in neuronal cells by glutamate and the enzyme glutamate decarboxylase (GAD). Reduction of GABA, normally acting as inhibitory mechanism in the central nervous system (CNS), can cause epileptic seizures as well as dystonia, as mentioned in a few publications (Plaitakis A. et al. 2013).

Increasing rate of published studies is relating the deregulation of glutamate homeostasis and astrocytes participation in the development of age related diseases, such as Alzheimer's disease (AD) (Kulijewicz - Nawrot M. et al., 2013, Olabarria M. et al., 2011).

In a study, published by Bao X. et al. on 2009, authors created a mouse model of enhanced glutamate release by using a system of over expression of GDH. Even though, it is not fully defined wether the over expression of GDH leads to upregulation or downregulation of glutamate in the neural tissue. That is because of the different tissue characteristics and variability in cell types and function. Bao and his colleagues found glutamate release enhanced, in specific brain regions in mice such as in striatum and hippocampal CA1 region. That finding was accompanied by finding that suggest progressive neurodegeneration in many brain areas of

transgenic mice. They ascertained a significant reduction in the number of the dendritic spines and axonal nerve terminals in CA1 hippocampal region. That finding of degeneration became more intense and was further represented by other brain regions, when animals got older. Authors suggested that GDH1 over-expression can accelerate age-related neuronal loss and dendritic dysfunction and that these changes in neural tissue are similar to those previously observed in Alzheimer's disease (Bao X. et al., 2009, Wang X. et al., 2014). These findings suggest that the action of GDH in neural tissue is something that needs further investigation in order to elucidate their specific roles that seem to be of great importance.

STUDIES OF HGDH OVEREXPRESSION IN CELLS

To our surprise, little information is provided in the literature regarding GDH over expression. There are some studies that tried to focus on that matter by searching the relationship of GDH with insulin secretion. In their experiments, the cells were transiently over expressing the enzymes. They over expressed GDH in mouse pancreatic cells using a retrovirus expression system for measuring the insulin secretion profile (Carobbio S. et al., 2004, Tanizawa Y. et al., 2002). These studies are focusing in that specific system because there are interested in the study of diabetes.

There are though, two papers focusing on neural tissue. These two studies, also mentioned earlier (section of neurologic disorders), were performed in mice, but this time they used transgenic mice modified to over express GDH1. They conclude that increased GDH expression in neural cells resulted in upregulation of glutamate release and consequently, excitotoxicity. The mice were exhibiting a phenotype similar to progressive neurodegeneration. Nevertheless researchers conclude that further studies need to be performed in order to clarify the cellular role and signaling pathways that GDHs affect. (Bao X. et al. 2009, Wang X. et al., 2014)

OBJECTIVES OF THE CURRENT STUDY

The objectives of this study concern two main questions, specifically, the question of the exact subcellular localization of the two isoenzymes and secondly, the cellular effect of their overexpression.

1) Concerning the first question, our goal was the study of the subcellular co-localization of the two iso-enzymes, namely hGDH1 and hGDH2. This part of the study was interesting to be performed because all the existing studies in the literature have only indirectly proven that both enzymes are localized inside mitochondria matrix, by using a GFP staining. Our study would be the first that applied different staining tags in each enzyme for their detection inside mammalian cells. Thus, this analysis would provide us with direct information about their subcellular localization.
2) Concerning the second question, our goal was to study the effect of hGDH1 and hGDH2 over-expression. To this aim, we aimed to focus on the construction of stable HEK293 cell lines, each of them over expressing either hGDH1 or hGDH2. After achieving the creation of the over expressing cell lines, we aimed to proceed with the analysis of the phenotypic characteristics these cell lines might present.

2. MATERIALS AND METHODS

DNA cloning

Our aim is to generate a plasmid construct that will express human *GLUD2* as a chimeric protein with mCherry tag (red) at its C-terminus. This construct will be used for the transfection of eukaryotic cells in order to easily distinguish the specific subcellular localization of our protein of interest-*GLUD2*. The cloning procedure consists of many different steps including: DNA digestion with restriction enzymes, ligation of DNA frogments of the vector and the insert, transformation of bacterial cells and expansion of positive clones, DNA purification and verification of the result and further expansion of the positive bacterial clone for high yields of purified DNA for subsequent use in mammalian cultures.

DIGESTIONS

Restriction endonucleases were used for two purposes in the present project: 1. for the excision of the pre-existing DNA fragment (mouse Caspr2 cDNA, kind gift from Dr Karagogeos lab) from pmCherry N1 plasmid vector and 2. for the generation of complementary endings in pmCherry N1 vector and double-stranded DNA segment (inserts) obtained by PCR from human Glud2 cDNA. The enzymes used were EcoRI which cuts upstream of the insert sequence, inside the multiple cloning site of the plasmid vector, and BamHI which recognizes a specific site downstream of the insert and upstream of the pmCherry sequence. Both recognition sites for the restriction enzymes are unique in the DNA sequence of the vector and are not detected either the insert or the pmCherry sequences. A digestion reaction consists of the DNA sample, the enzyme and its specific buffer as well as BSA for a group of enzymes (Bovine serum albumin, is used to prevent adhesion of the enzyme to reaction tubes and pipette surfaces. It also stabilizes some proteins during incubation). The temperature of the reaction id 37°C for most of the enzymes and it takes around 1,5 hrs for the reaction to be completed. In order to test the result of a digestion, a small volume is loaded in an agarose gel with gel-red (10000x). The consistency of the gel in agarose depends on the size of the DNA sample we need to visualize. Loading buffers are also used for the dilution of the sample, that help in increasing its density so that the sample loads correctly into the well. DNA ladder of

known molecular weights is also loaded in a different well, that makes easier the size prediction of the linear DNA bands.

The target bands were extracted from the agarose gel using DNA extraction kit by MACHEREY-NAGEL, following the provided protocol.

Polymerase Chain Reaction (PCR)

The technique used for obtaining the cDNA of *GLUD1* and *GLUD2* genes from plasmid vector pcDNA,3.1 was Polymerase Chain Reaction (PCR). PCR was the performed for cloning the selected gene and introducing new restriction sites on 3' and 5' of the generated DNA piece. On the 5' arm we generated EcoRI restriction site and on the 3' arm BamHI restriction site. The restriction sites were introduced in our product with designed primers. The upstream primer contained the restriction site EcoRI sequence, as well as homology part of ~15 nucleotides of our target gene. The downstream primer contained homology part ~15 nucleotides, a point mutation that modifies the stop codon in a neutral amino acid and finally the BamHI restriction site sequence.

PRIMERS

EcoRI-GLUD2 sense 5-CCGGAATTCATGTACCGCTACCTGGCC-3

GLUD1/2 BamHI ANTI-SENSE: 5-ATTCGCGGATCCCCCTTTGTGAAGGTCACACCA-3

PCR Reaction

Buffer (Kapa polymerase) 5x → 4µl

MgCl (25mM) → 1µl

dNTPs (2mM) → 2µl

primer Forward EcoRI – GLUD2 (5pmol/µl) →1 µl

primer Reverse LM_BamHI (5pmol/µl) →1 µl

Taq DNA polymerase Kapa (5u/µl) →0,25 µl

DNA template (pc DNA,3.1-GLUD1/ pc DNA,3.1-GLUD2)(~150ng)→1,5µl

H2O → 9,25µl

Total volume per sample →20µl

PCR PROGRAMME

95°C for 5 minutes

95°C for 1 minute
56°C for 1 minute ⇨ 35 cycles
72°C for 1 minute

72°C for 10 minutes

4°C for ∞

- 2 µl of the PCR products were loaded on 1% agarose gels with gel red on 100V for verification of the results.
- The verified products were cleaned up using PCR clean up kit from MACHEREY-NAGEL following the provided protocol.

LIGATION

The next step in cloning is ligation. Ligation is the junction of 2 or more DNA fragments that have complementary or blunted endings. The enzyme used for this purpose is T4 DNA ligase that originates from *Escherichia coli* bacterial strain, which contain plasmids with gene encoding this enzyme at high expression levels. T4 DNA ligase needs ATP and Mg+2 as co-factors for its function which is to generate bonds between 3'-OH and 5'-P of free DNA endings. Various molecular ratios are tested for the best outcome, with the most usual being 5:1, 3:1 and 2:1, insert:vector. The reaction contains the DNA fragments to be ligated, the enzyme and its appropriate buffer, and the temperature is sometimes suggested by the manufacturer of the enzyme. 20-25° C were used for the present protocol.

After ligation, the plasmid construct is inserted with transformation in *Escherichia coli* bacteria of the DH10b strain.

TRANSFORMATION

The transformation method that was selected is electroporation. In order to generate electrocompetent cells, 100ml of LB* medium (1lt: 5gr yeast extract, 10gr bactotryptone, 2gr NaCl, pH 7.0) were inoculated with DH10b cells at 37^0 C under shaking until the optical density of the culture reaches 0.6 at 600 nm.

Afterwards:

1. Cells are incubated on ice for 15΄

2. Centriguge at 4000 rpm, 4°C for 15΄

3. Remove supernatant and dissolve the bacterial pellet in 100ml of cold nanopure H_2O

4. Centriguge at 4000 rpm, 4°C for 15΄

5. Remove supernatant and dissolve the bacterial pellet in 50ml of cold nanopure H_2O 6. Centriguge at 4000 rpm, 4°C for 15΄

7. Remove supernatant and dissolve the bacterial pellet in 25ml of cold nanopure H_2O

8. Centriguge at 4000 rpm, 4°C for 15΄

9. Remove supernatant and dissolve the bacterial pellet in 10ml of cold nanopure H_2O

10. Centriguge at 3500 rpm, 4°C for 15΄

11. Remove supernatant and dissolve the pellet in ~1ml κρύο 10% v/v glycerol solution

12. Electrocompetent cells can now be used or can be kept in aliquots at -80°C

The above-mentioned procedures take place on ice. For the electroporation, 1-2μl of the ligation reaction are added into the cells and mixed. Subsequently, the mix is transferred in cold cuvette and electroporation is applied (1750 V για 5msec).

Afterwards, ~800µl of 2XYT medium (1lt: 16gr tryptone, 10gr yeast extract, 10gr NaCl, pH 7.2-7.4) are added to the cells, and incubation occurs at 37°C for 1hr under shaking. Finally cells are plated on a petri dish with LB medium (1lt: 5gr yeast extract, 10gr bactotryptone, 10gr NaCl, pH 7.0), agar (12gr for 1lt of medium) and the appropriate selection antibiotic.

PLASMID DNA PURIFICATION (MINI PREP) PROTOCOL FROM MACHEREY-NAGEL

1. Cultivate and harvest bacterial cells (12,000 x g, 30 s)

2. Cell lysis

150 µL Buffer A1

250 µL Buffer A2

RT, up to 2 min

350 µL Buffer A3

3. Clarification of the lysate (> 12,000 x g, 3 min)

4. Bind DNA Load supernatant (1,000–2,000 x g, 30 s)

5. Wash and dry silica membrane

450 µL Buffer AQ

> 12,000 x g, 1 min

6. Elute DNA with 50 µL Buffer AE, RT, 1 min > 12,000 x g

PLASMID DNA PURIFICATION (MIDI PREP) PROTOCOL FROM MACHEREY-NAGEL

Cultivate and harvest bacterial cells (4,500–6,000 x g, 4 °C, 15 min)

Cell lysis

8 mL Buffer RES

8 mL Buffer LYS, RT for 5 minutes

Equilibration of the column and filter with 12 mL Buffer EQU

Neutralization using 8 mL Buffer NEU, Mix by gently inverting 10–15 times

Clarification and loading of the lysate, Invert the tube 3 times

Load lysate on NucleoBond® Xtra Column Filter

1st Washing 5 mL Buffer EQU

Discard NucleoBond® Xtra Column Filter

2nd Washing 8 mL Buffer WASH

Elution 5 mL Buffer ELU

Precipitation 3.5 mL Isopropanol 15,000 x g, 4 °C, 30 min

Wash and dry DNA pellet using 2 mL 70 % ethanol, 15,000 x g, RT, 10 min

Reconstitute DNA in appropriate volume of ddH2O, store at -20°C

TRANSFECTION OF MAMMALIAN CELL CULTURES

Transfection of mammalian cells with plasmids expressing GDH1, GDH2, fluorescent proteins or chimeric expression of GDH 1 or 2 with a fluorescence protein were performed using lipofectamine 2000 provided by invitrogen.

- the cells were cultured in tissue culture dishes of 60mm size or six wells plates in a medium containing DMEM with 10% fetal bovine serum and the appropriate antibiotics
- when the cell culture succeeded 70 – 90 % confluency they were ready for transfection
- the day of transfection the medium was replaced by fresh medium without antibiotics
- 4 hours later, for each plate to be transfected was diluted in 250µl Opti-MEM medium 5 – 11 µg DNA plasmid in a 1,5ml tube. In another tube were diluted 250 µl Opti-MEM with 2,5 – 3 times lipofectamine 2000 the amount of µg DNA plasmid added.
- Mix the content of the two tubes together an incubate at RT for 20 to 25 minutes

- After the incubation the DNA – lipid mixture is slowly and carefully added to the cultured cell dish.
- 4 hours later to 24h later the medium is replaced by fresh without antibiotics for toxicity from lipofectamine to be avoided
- 24 to 72 hours later the transfected cells are ready for analysis

CREATING STABLE EXPRESSION OF HEK293 CELL LINES

1. Transfect mammalian HEK293 (Human Embryonic Kidney) host cell line with your pcDNA™3.1 construct containing *GLUD1* or *GLUD2* gene using the transfection protocol.

- The constructs used for creation of stable cell lines were

  pcDNA 3,1- *GLUD1*

  pcDNA3,1 – *GLUD2*

  GLUD1-eGFP

  GLUD2-eGFP

  peGFP

2. 24 hours after transfection, wash the cells and add fresh medium without antibiotics to the cells.

3. 48 hours after transfection, split the cells into fresh medium containing 0,6 mg/ml GeneticinR required for your cell line. Split the cells such that they are no more than 25% confluent.

4. Split the cells every 24 hours so that every cell is exposed to the antibiotic

5. Feed the cells with selective medium every 3–4 days until Geneticin R-resistant foci can be identified.

6. Pick and expand colonies in 96- or 48-well plates

7. Analysis and characterization of the cells stable expressing the preferable protein

Mitochondria Isolation Kit for Cultured Cells

The kit was provided from thermo scientific and it was used for the isolation of mitochondria from mammalian cultured cells. Specifically the method used for the samples of our study was the reagent-based method.

Isolation of Mitochondria using Reagent-based Method

• Immediately before use, add protease inhibitors to Reagent A and Reagent C; only add inhibitors to the reagent amount being used for the procedure and not to the stock solutions.

• Process up to six samples concurrently.

• Required speed of vortex changes during the protocol.

1. Pellet 2 × 107 cells by centrifuging harvested cell suspension in a 2.0mL microcentrifuge tube at ~850 × *g* for 2 minutes. Carefully remove and discard the supernatant.

2. Add 800µL of Mitochondria Isolation Reagent A. Vortex at **medium** speed for 5 seconds and incubate tube on ice for exactly 2 minutes.

Note: Do not exceed the 2 minute incubation.

3. Add 10µL of Mitochondria Isolation Reagent B. Vortex at **maximum** speed for 5 seconds.

4. Incubate tube on ice for 5 minutes, vortexing at **maximum** speed every minute.

5. Add 800µL of Mitochondria Isolation Reagent C. Invert tube several times to mix (**do not vortex**).

6. Centrifuge tube at 700 × *g* for 10 minutes at 4°C.

7. Transfer the supernatant to a new, 2.0mL tube and centrifuge at 12,000 × *g* for 15 minutes at 4°C.

Note: To obtain a more purified fraction of mitochondria, with > 50% reduction of lysosomal and peroxisomal contaminants, centrifuge at 3000 × *g* for 15 minutes.

8. Transfer the supernatant (cytosol fraction) to a new tube. The pellet contains the isolated mitochondria.

9. Add 500µL Mitochondria Isolation Reagent C to the pellet, and centrifuge at 12,000 × *g* for 5 minutes. Discard the supernatant.

10. Maintain the mitochondrial pellet on ice before downstream processing. Freezing and thawing may compromise mitochondria integrity.

- After the successful mitochondrial fraction isolation obtained, buffer H + 1% triton for 10 minutes (vortex every minute) was added to the samples for the mitochondria membrane lysis.
- Final step was centrifuge of the samples at high speed for 2 minutes so that the supernatant contains soluble mitochondria protein without the membranes

WESTERN BLOT ANALYSIS

- Split the culture flasks of mammalian cells HEK293 and collect the medium with the cells.
- Centrifuge the LB medium at 1500rpm for 10 minutes in order to dissociate the cell pellet.
- Resuspend the cell pellet in 250 µl lysis buffer containing Buffer H + 1% triton + protease inhibitor 100x by pipetting up and down thoroughly.
- Place on ice for 60 minutes.
- Centrifuge at 11.000g for 10 minutes and collect the supernatant which contains the proteins of the cells
- Preparation of 1,5mm-thick well gels for loading and running the samples

Resolving gel buffer	*Stacking gel buffer*
1.5 Tris-HCl pH 8.8 (150 ml)	**0.5 M Tris-HCl ph 6.8 (100 ml)**
27.23 gr Tris base	6 gr Tris base
80 ml dd H2O	60 ml dd H2O
adjust to pH 8.8 with 6N HCl	adjust to pH 6.8 with 6N HCl
bring total volume to 150 ml with ddH2O	bring total volume to 100 ml
store at 4 C	store at 4 C

RESOLVING GEL 10%
ddH2O 4.1 ml
30% bis/acrylamide 3.3 ml
gel buffer *1.5 Tris-HCl ph 8.8 2.5 ml
10% SDS 100 µl
10% APS 50 µl
TEMED 5 µl

STACKING GEL 4%
ddH2O 6.1 ml
30% bis/acrylamide 1.3 ml
gel buffer *0.5 Tris-HCl ph 6.8 2.5 ml
10% SDS 100 µl
10% APS 50 µl
TEMED 10 µl

- Bradford dye was used for the quantification of the concentration of total protein in the samples
- After determining the protein sample volume add 4X loading dye with 4% mercaptoethanol and 1X loading dye up to 40µl
- heat samples at 95 C for 4-5 min and keep samples on ice
- load samples and the marker on the gels after placing them on device provided by biorad
- add about 1 L of 1x running buffer
- run at 100V through the stacking gel (approx.30 min) and then set to 150 V until the dye runs out (approx.2 h)

10x running buffer (1 L pH 8.3)

30.3 gr Tris base

144 gr glycine

10 gr SDS

dissolve and bring total volume up to 1000 ml with dd H20

store at 4 C

Transfer

- cut out filter papers and membrane (6 wattman pieces, 1 membrane for each gel)
- dispose running buffer from gel apparatus
- remove the stacking gel in a tray containing transfer buffer and on the cassette place a sponge, then 3 pcs of filter paper, the gel, the membrane and then again 3 pcs of filter paper and finally the last sponge.
- Place the cassette on the transfer devise
- Fill the container with ice-cold transfer buffer (add additional ice-packs if necessary)
- Run at 350mA for 1h

transfer buffer (place the buffer at 4 C at least 2hrs before the transfer take place)

for 1000 ml 1x transfer buffer

100 ml 10x running buffer

700 ml dd H2O

200 ml MethOH

Immunoblotting

- remove membrane from sandwich, place it on a rack and block it in 5% milk for 1hr (on a shaker)

5% blocking

2.5 gr milk powder – up to 50 ml 1x PBS TWEEN

- remove blocking solution
- incubate your membrane with primary Ab diluted in 5% milk in pbs tween, make sure that the whole membrane is covered (~12 ml)
- incubation O/N – 4 C – on a shaker.
- remove Ab and quickly wash with 1x PBS – 3 X

5 then wash with PBS-tween – 3 X (15 min, 5 min, 5 min)

- prepare secondary Ab and incubate for 1hr – RT – on a shaker

- Next discard Ab and quick wash with 1X PBS – 3 x
- then wash with 1X PBS-tween – 3 X (15 min, 5 min, 5 min)
- wash twice with 1X PBS – 2x5 min
- Use ECL kit developing the results on film (1.5 ml in total : 750 µl of each reagent – do not forget to mix well before applying)

IMMUNOCYTOCHEMISTRY PROTOCOL

1st day

1. Sterilize coverslips under UV light for 15min
2. Grow cells on coverslips

2nd day

3. Fix with paraformaldehyde (PFA) for 15min at RT
4. Rinse twice with 1ml PBS at RT
5. Blocking with PBS-TritonX100 0,5% with 2%NGS (normal goat serum), 500λ in each well for 1h at RT
6. Immediately, no rinse-just discard the blocking, add the first Ab. The Ab should be prepared in the same medium that was used for blocking. 200λ in each well. (Antibodies for hGDH1 & hGDH2 1:5000 dilution). Incubate overnight at 4 C.

3rd day

7. Wash with PBS 3*8min at RT
8. Incubate with appropriate amount of 2nd Ab for 1h. (biotinylated anti-rabbit 1:200)
9. Wash with PBS 3*5min at RT
10. Incubate with appropriate amount of fluorophore for 1h
11. Wash with PBS 3*5min at RT
12. Incubate with TO-PRO for 3min (1:1000 in PBS)

13. Mount coverslip with a drop of mounting medium.

14. Seal coverslip with nail polish to prevent drying and movement under microscope

15. Store in the dark at +4°C.

DNA FRAGMENTATION ANALYSIS USING AGAROSE GEL ELECTROPHORESIS

A. The first step is the purification of genomic DNA from eukaryotic cultured cells using proteinase K, a protocol from Thermo scientific.

The following protocol is used for 1-2 × 10^6 cells.

1. Centrifuge for 5 minutes at 3,000 rpm in a microcentrifuge to collect the cells.

2. Wash the pellet twice with PBS

137 mM NaCl

27 mM KCl

100 mM Na2HPO4

2 mM K2HPO4

(pH 7.4)

3. Resuspend the pellet in 0.5 mL of **lysis buffer**

10 mM Tris-HCl, pH 8.5

5 mM EDTA

200 mM NaCl

0.2% SDS

Incubate at 60 °C for 5 minutes

4. Add 2.5 µL of Thermo Scientific Proteinase K and 5 µL of Thermo Scientific RNase mix. Incubate at 60 °C for 1 hour.

5. Add 250 µL of 5 M NaCl, mix and incubate on ice for 5 minutes to precipitate protein.

6. Centrifuge for 15 minutes at 10,000 rpm in a microcentrifuge.

7. Transfer supernatant to a fresh tube. Add an equal volume of isopropanol and mix to precipitate the DNA.

8. Optional. Incubate at -20 °C for up to 60 minutes to increase the yield of DNA.

9. Centrifuge for 10 minutes at 10,000 rpm in a microcentrifuge.

10. Discard the supernatant and wash the pellet with 1.2 mL 70% cold ethanol.

11. Air-dry the pellet and dissolve the pellet in Water, nuclease-free or TE buffer.

Note: The typical yield of DNA from 106 cells is 7-15 µg.

B. LOAD SAMPLES IN AGAROSE GEL ELECTROPHORESIS

1. Add 5 µl of 6X DNA loading buffer and load DNA samples into dry wells of a 1-1.5% agarose gel in TAE containing 0.5 µg/ml Gel Red.

2. Run the gel at a low voltage, which improves resolution of DNA fragments

3. DNA ladders are finally visualized by a UV light source and documented by photography. Apoptotic cells will form a distinct DNA ladder, whereas necrotic samples may generate a smear pattern (or no DNA fragmentation). DNA from viable cells will stay on the top of the gel as a high-molecular-weight band.

3. RESULTS

Construction of a plasmid expressing chimeric *hGDH1* with red fluorescence protein using mutated pmCherry vector

The construct was performed to be used for the study the co-localization of the two iso-enzymes hGDH1 and hGDH2. Our laboratory already possessed a plasmid construct of the GLUD1 gene (cDNA) expressing chimerically hGDH1 with a green fluorescence protein (peGFP), (Mastorodemos et al., 2009). The main project idea was to create another construct that could chimerically express one Red Fluorescence Protein (RFP) conjugated with hGDH2 for the detection of the enzyme inside mammalian cells with the help of confocal microscopy. That way, after proceeding in co-transfection of the two plasmids in mammalian cells, we would be able to detect the subcellular localization of the enzymes using an SP2 confocal microscope. With this method, the observer is able to detect the localization of the proteins in specific compartments of the cells, as well as the whether they co-localize or not.

The first plasmid selected for the construction of chimerical expression of *GLUD2* gene with Red Fluoresence Protein was pdsRed-mito. The initial design was rejected due to the difficulty we faced when tried to remove the mitochondrial sequence from the plasmid and ligate the arms.

The construct was re-designed from scratch by using a different vector, in that case, pmCherry who was obtained from Dr Karagogeos lab. The vector was already currying casper2 protein gene inside the plasmid's multiple cloning site. It was introduced in the plasmid by using EcoRI restriction site on the 5' arm and BamHI on the 3' arm. The same restriction enzymes used for removing casper2 gene from the plasmid. In the figure below, a detailed map of the vector is provided, selected for fulfilling the project's goal.

pmCherry-N1 Vector Information

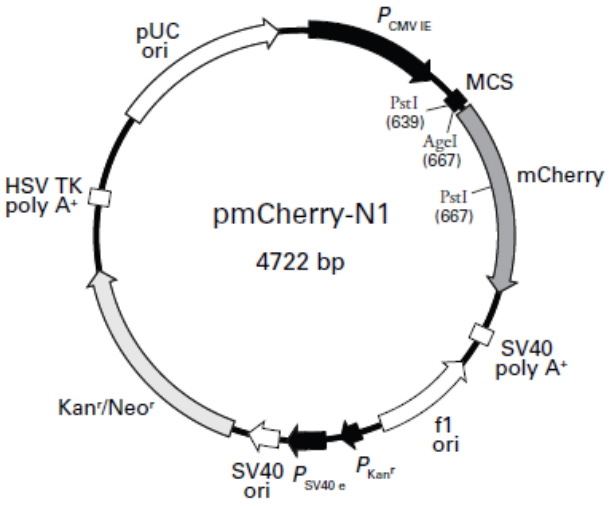

Fig 3.1 The map of the plasmid vector used

The first step was the purification of the plasmid from *E. coli* bacterial cells in midi prep scale, using a kit provided by Macherey-Nagel (Valencienner Str. 11, Düren, Germany). After the purification, we proceeded in quantitation of the DNA purified by using spectophotometry (nano drop). The plasmid DNA was diluted in concentration of 1.7 μg/μl in a total volume of approximately 500μl.

After the purified plasmid was obtained, the next step was to perform double digestions using the restriction enzymes EcoRI and BamHI (37°C, ~2hours), for removing the Casper2 gene of 3600 base pairs total size and achieve a purified vector that should be able to accept our insert, *GLUD2* cDNA.

Following double digestion, the samples were loaded in a big scale agarose gel 0.6% for dissociation of the sample bands by size. The gel was running at 70V for approximately 9 hours. We faced a difficulty separating the two bands on the gel because the two pieces of the plasmids had small differences in size. (Fig. 3.2)

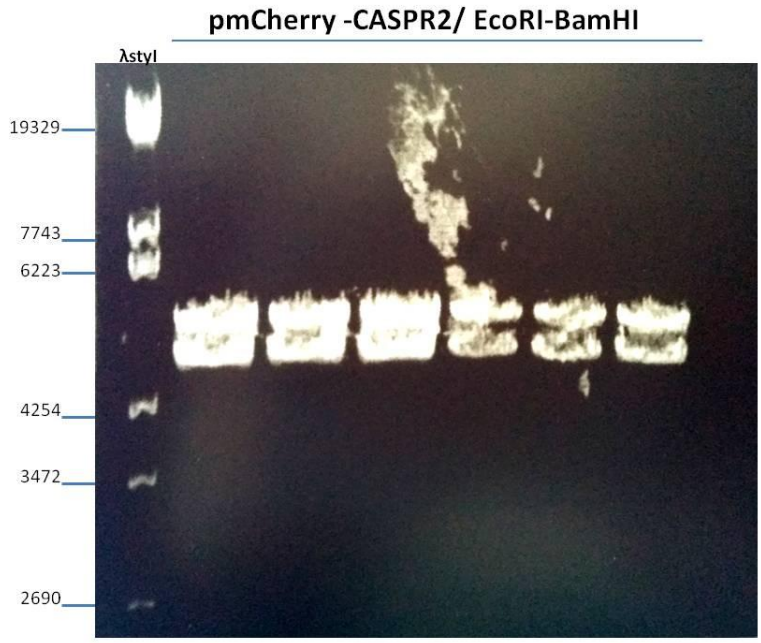

Fig 3.2 : Photo of an agarose gel showing the two bands of DNA seperated after double digestion using the enzymes EcoRI/BamHI and electrophoresis. Upper band is the vector and the lower band is the Caspr2 insert

Then, the bands close to 4700bps were obtained from the agarose gel.

Fig 3.3: The same agarose gel presented in (fig. 3.2) after the vector extraction was performed

The products were extracted from the gel using a gel extraction kit from Macherey-Nagel and diluted in 20µl of TE buffer. Stored at -20°C for future use. The plasmid vector was now ready for the introduction of *GLUD2* gene.

The detailed map of the designed final product is in the figure below named pmCherry-*GLUD2*. (fig. 3.4)

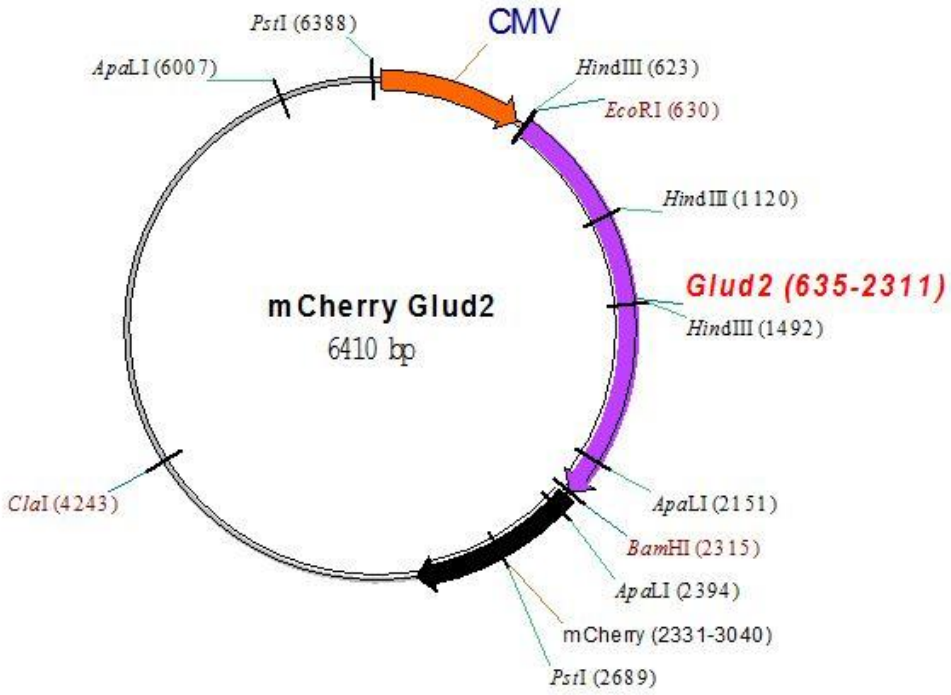

Fig 3.4 : A detailed map of the designed final construct plasmid including *GLUD2* gene

The next step of the construct procedure was to create the insert (*GLUD2* cDNA). That could be achieved by using PCR method. Specifically, the selected primers were designed for introducing an EcoRI restriction site in the forward primer and restriction site BamHI in the reverse primer. In the reverse primer, we also introduced several nucleotides for correcting the reading frame and a point mutation replacing the stop codon with another amino acid. This enabled us to obtain the chimeric protein (hGDH2 fused with RFP).

The PCR reaction was performed using the compounds and the PCR machine program that are described in the section materials and methods.

- 2 µl of the PCR products were loaded on 1% agarose gels with gel red on 100V for verification of the results.
- The verified products were cleaned up using PCR clean up kit from MACHEREY-NAGEL following the manufacturer protocol.

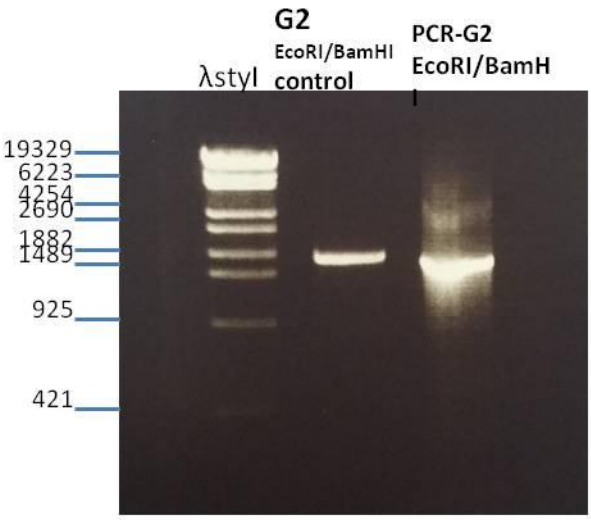

Fig 3.5 : Photo of an agarose gel where we loaded the *GLUD2* insert PCR product (last lane) and, for comparison, a control *GLUD2* cDNA (second lane) cut using the restriction enzymes EcoRI/BamHI

The mutated insert and the vector were loaded in 1% agarose gel for rough quantitation before proceeding in the ligation step.

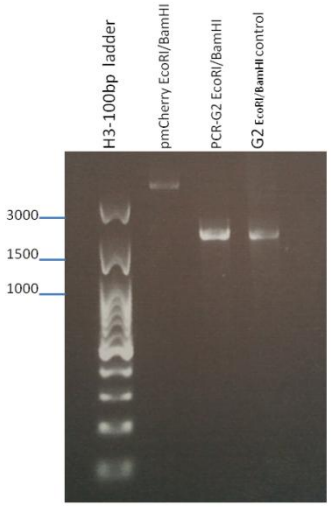

Fig 3.6: Photo of an agarose gel where equivalent volumes of PCR reaction from both insert *GLUD2* (third lane) and vector pmCherry (second lane) were loaded for quantification. On the first lane the marker H3-100bp ladder is loaded. On the last lane a control of *GLUD2* cDNA cut using enzymes EcoRI/BamHI is loaded.

The ligation reaction was performed and incubated over night at room temperature. The next day the ligation product was transformed in electro competent e-coli, bacteria using electroporation. The e-coli with the ligation product were electroporated at 2100V for 5ms. Following the protocol, the bacteria liquid culture was plated in agar plates with kanamycin (30-50 ng/ml). The plates were incubated in a sterile incubator, over night at 37°C. The following day the positive colonies were purified using mini preps kit from Macherey-Nagel.

The colonies were characterized through digestion analysis, using multiple restriction enzymes combinations.

For example using the restriction enzymes EcoRI and BamHI were the insert was introduced in the vector.

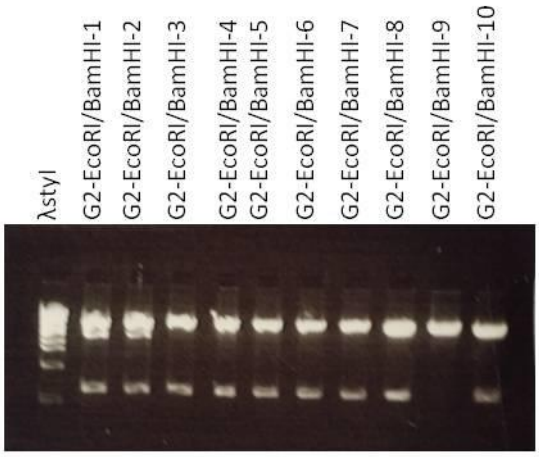

Fig 3.7: 10 recombinant plasmid samples after the ligation step was performed using insert *GLUD2* with vector pmCherry. The DNA samples were purified from colonies selected by the agar plate for characterization using digestion analysis. The restriction enzymes used were EcoRI/BamHI, for distinguishing the colonies that the insert was introduced.

Moreover, for digestion analysis we used the restriction enzymes SalI and EcoRI for verification of *GLUD2* gene instead of *GLUD1*.

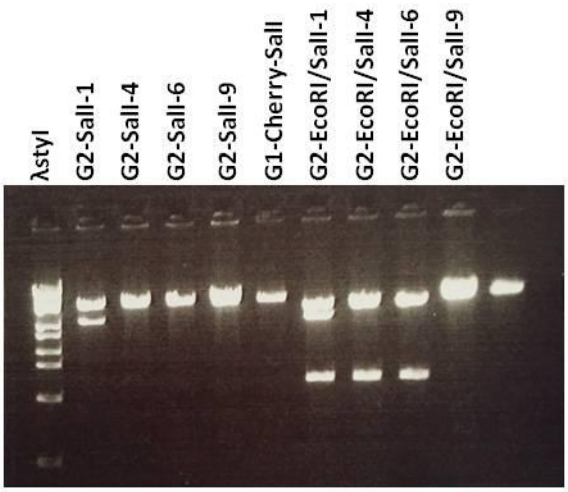

Fig 3.8 : Photo of an agarose gel after digestion analysis for further characterization of colonies selected from previous step (fig. 3.7) fulfilling the criteria of successful recombination of the insert *GLUD2* introduced in vector pmCherry. In half of the samples, we performed digestion using SalI only (lanes 2-6). In the other five lanes (7-11) double digestions were performed using restriction enzymes, EcoRI and SalI.

The most promising colonies were selected and sent for sequencing analysis. This sequencing analysis revealed that all the colonies selected had incorporated the insert, but in all of them we could recognize at least a single point mutation, probably as an error from PCR k polymerase used. For the reason that in our experiments, there is no need for fully functional enzyme we selected a colony with a point mutation far away from the leader peptide. That should have no effect in the localization of the protein which we want to study.

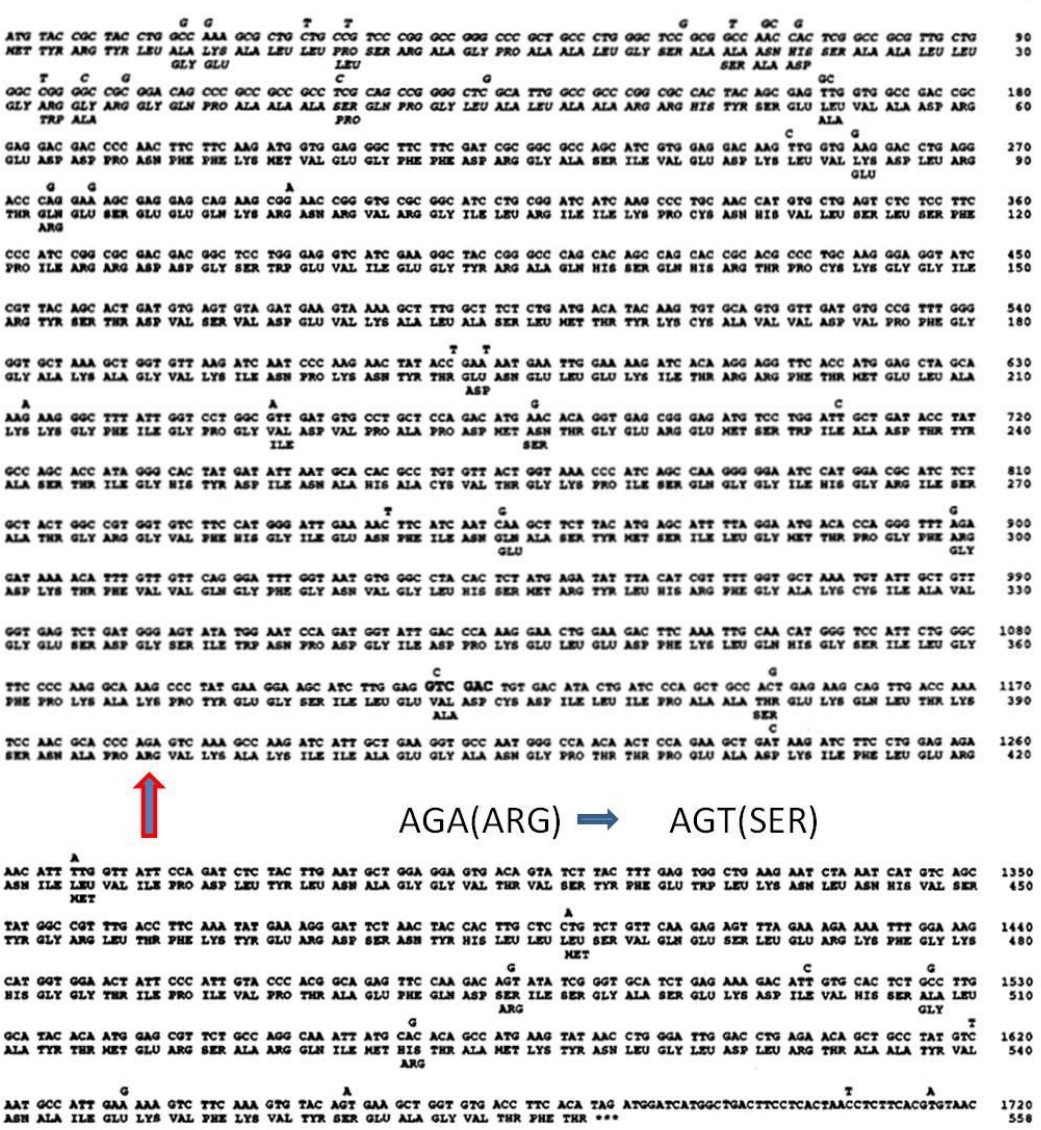

Fig 3.9 The sequence of the construct *GLUD2*-pmCherry as achieved after sequencing performed by MACROGEN Corporation. A sense point mutation was detected inside the *GLUD2* sequence; hopefully not affecting our results because it is located far from the leader peptide of the enzyme.

That product was then co-transfected in human embryonic kidney cells (HEK293) and in human HeLa cells. The products transfected were *GLUD1*-eGFP and pmCherry-*GLUD2* using lipofectamine 2000. The cells after the expression time needed were fixed with PFA in slides and visualized in SP2 confocal microscope.

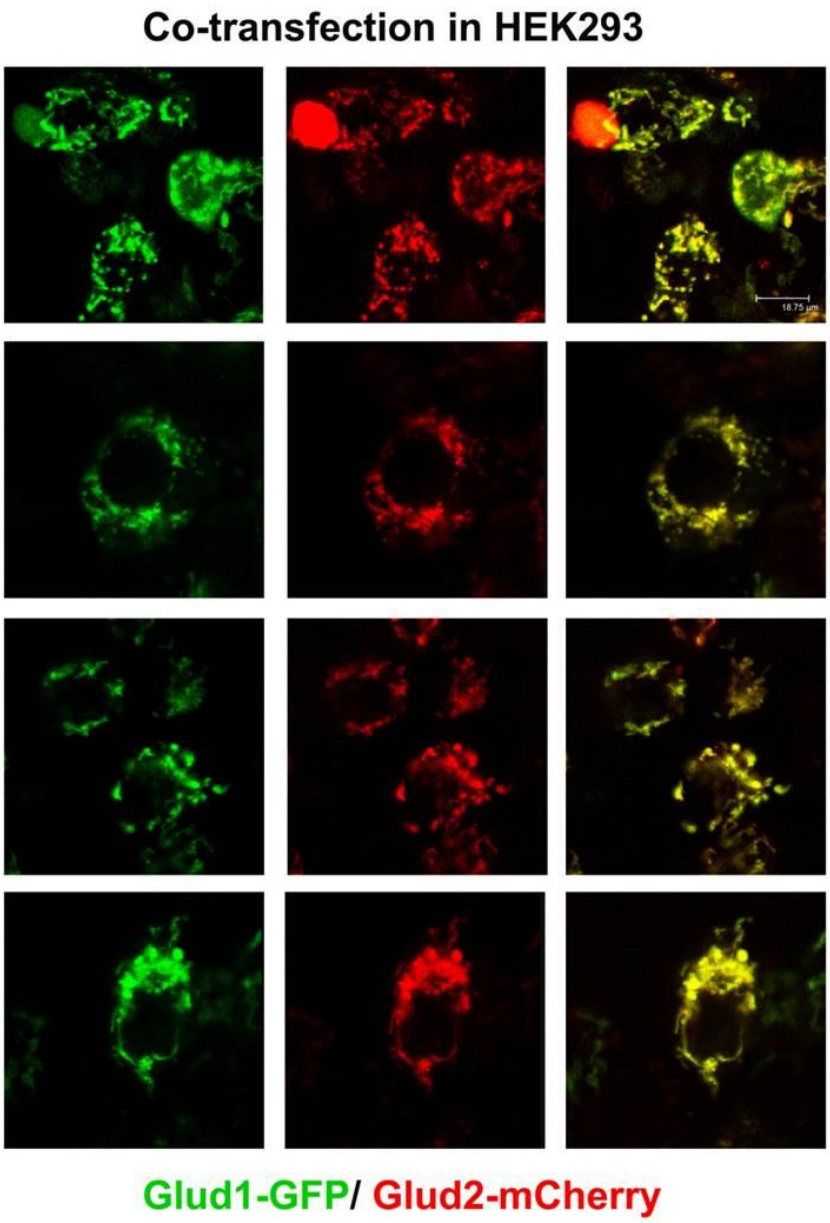

Fig 3.10. Confocal imaging of co-transfection in HEK293 cells using *GLUD1*-eGFP and *GLUD2*-pmCherry constructs. *GLUD1* tagged with green (GFP) is in the first lane. *GLUD2* tagged with red (RFP) is in the second lane. In the third lane we can see the merged signal of the two fluorescence proteins. The two enzymes seem to co-localize in the mitochondria.

The same co-transfection results from HeLa cells also.

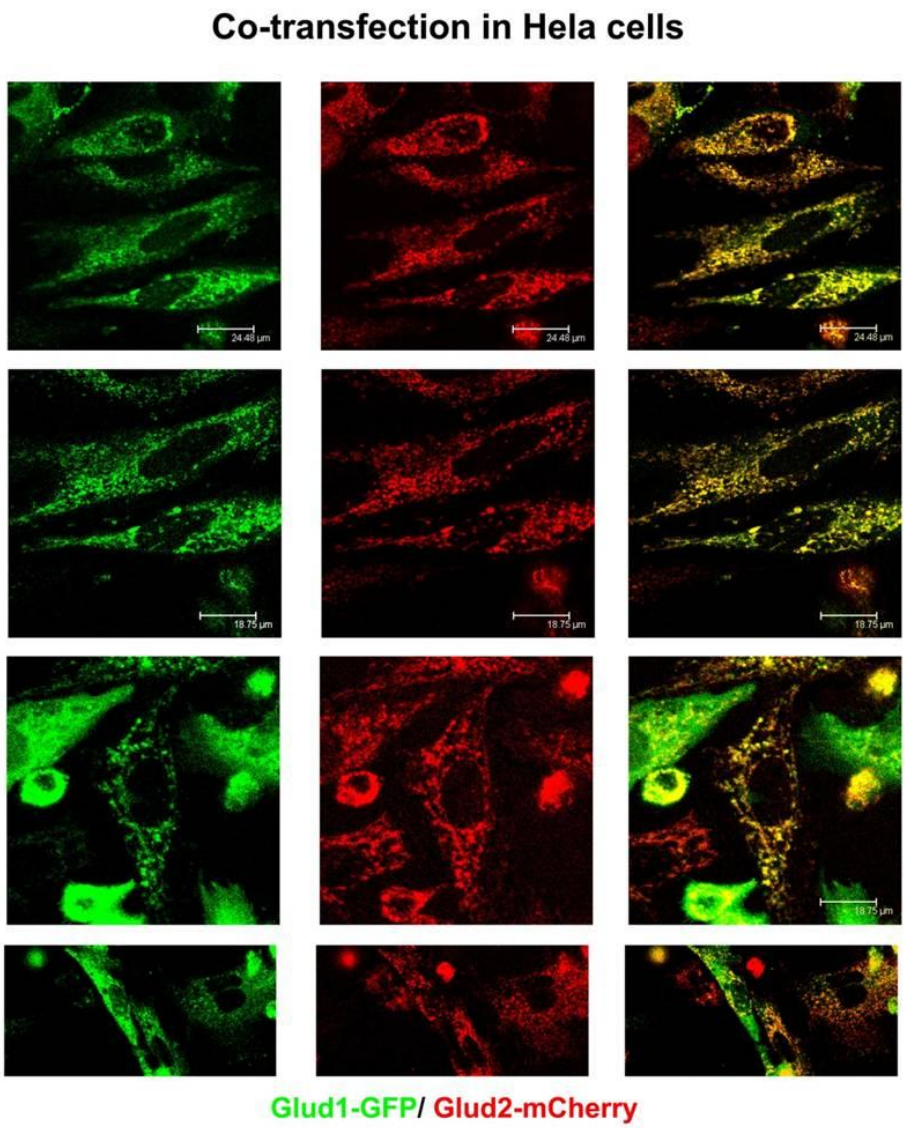

Fig 3.11. Confocal imaging of co-transfection in HELA cells using *GLUD1*-eGFP and *GLUD2*-pmCherry constructs. *GLUD1* tagged with green (GFP) is in the first lane. *GLUD2* tagged with red (RFP) is in the second lane. In the third lane we can see the merged signal of the two fluorescence proteins. The two enzymes seem to co-localize in the mitochondria.

As concluded from the pictures and the results obtained from confocal microscopy we can claim that hGDH1 and hGDH2 are co-localized in the cells and that localization is observed in the mitochondria as already published (Mastorodemos et al., 2009). That conclusion is provided by the characteristic staining of the mitochondria in cells as well as, from the merge signal that shows the co- localization of the two enzymes in the cells.

CREATION AND STUDY OF HEK 293 STABLE CELL LINES OVER EXPRESSING hGDH1 AND hGDH2

Many questions in cellular biology are often answered when the target protein or enzyme studied is over expressed in cells. This is because in that way researchers are able to keep all the parameters unchanged except the target protein levels which may vary from over expression to silence. That way it can be studied what consequences the alternate protein levels may cause to the cells.

In this case, our goal is to study the function and activity of those two isoenzymes inside human living organism. Because that goal is impossible to happen at this point, it may be a part of it achieved, if we manage to generate appropriate and successful cell lines that could over express our enzymes.

For that project we used a protocol from Invitrogen for the generation of stable cell lines using HEK 293 cells. After the construction of multiple cell lines for the verification and standardization of the technique, we created stable cell lines over expressing the enzymes, two colonies for hGDH1 and another two for hGDH2.

B.1 CHARACTERIZATION EXPERIMENTS FOR THE VERIFICATION OF OVER EXPRESSION IN HEK 293 CELLS

At this point of the study what needed was to verify that our cells are over expressing the enzymes. For the verification of this finding we used multiple ways and techniques.

IMMUNOCYTOCHEMISTRY

One or the first techniques used was immunocytochemistry using specific antibodies designed for either hGDH1 or hGDH2. By immunocytochemistry we obtained these results using SP2 confocal microscope.

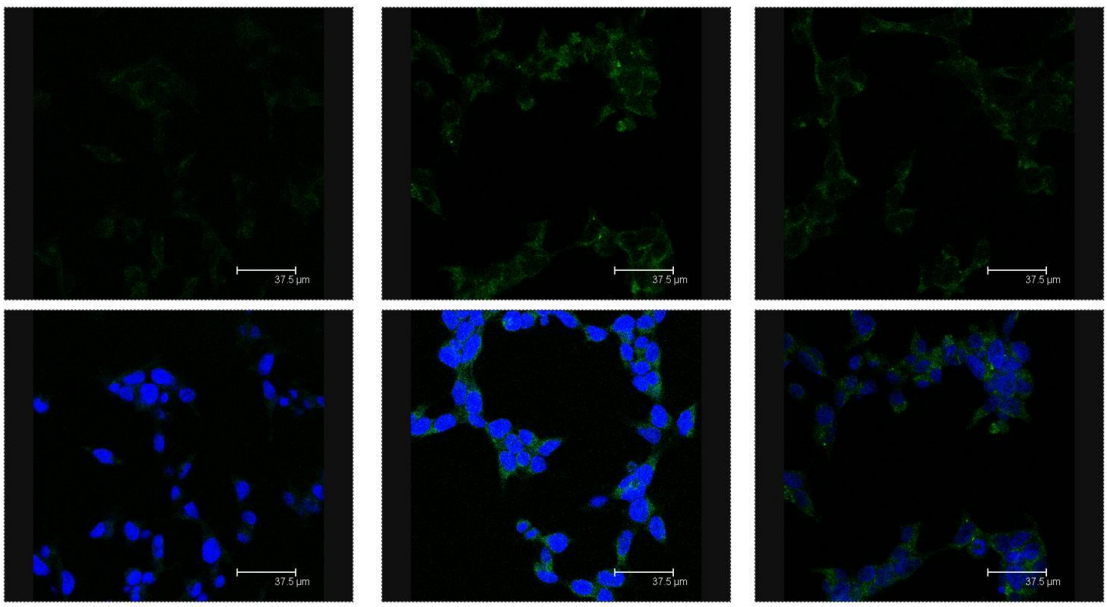

Fig 3.12. Immunocytochemistry using specific antibody for hGDH1 in WT HEK293 and two colonies of stable cell lines over expressing hGDH1. TOPRO3 is used for nuclei staining and is depicted in blue. The same laser intensity was applied in all cases during confocal microscopy

In the figure 3.12 we used specific antibody for hGDH1 in WT HEK293 and in our two colonies of stable cells (col2 and col6). The exposure of the signal was the same in all the samples for maintaining the ability to compare the samples between them. As we can observe the signal of the stable cells on both colonies is slightly increased.

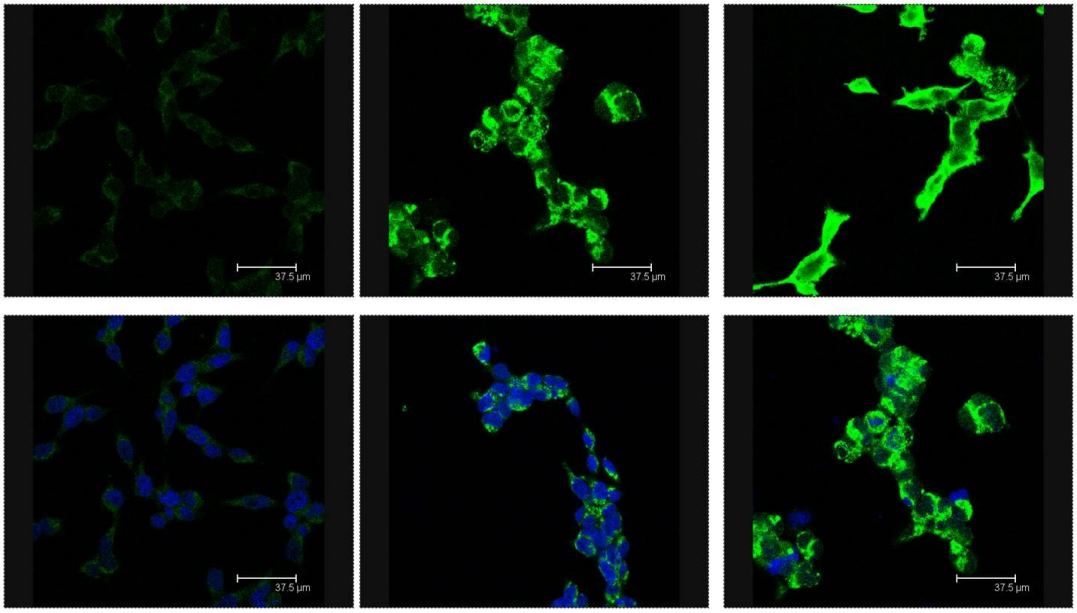

Fig 3.13. Immunocytochemistry using specific antibody for hGDH2 in WT HEK293 and two colonies of stable cell lines over expressing hGDH2. TOPRO3 is used for nuclei staining and is depicted in blue. The same laser intensity was applied in all cases during confocal microscopy

The same results can be observed in stable cells over expressing hGDH2 using antibody specific for hGDH2 (fig. 3.13); although this times the difference in the signal intensity was bigger.

WESTERN BLOT ANALYSIS

One other way chosen for the verification of our results was western blot analysis, using the same specific antibodies for hGDH1 and hGDH2. We performed several experiments for the verification of our results. In the first plot we loaded the whole lysate from the colonies for the detection of possible differences.

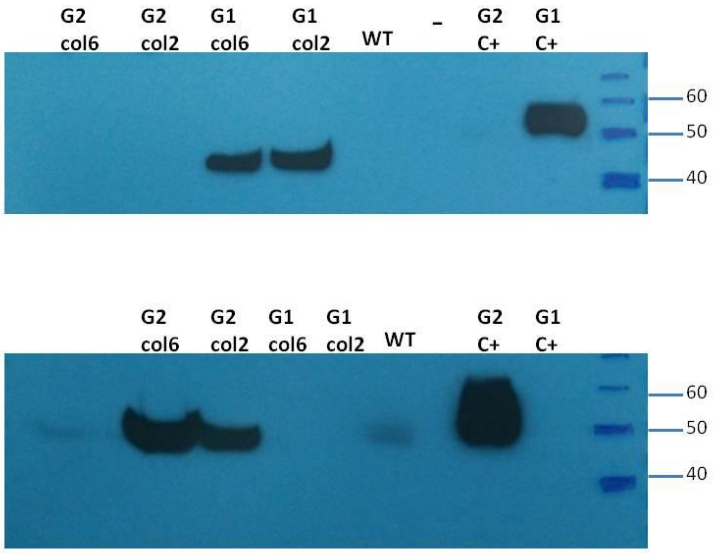

Fig 3.14. Western blot analysis of whole cell lysates from WT and stable cell lines colonies (col2 and col6). The blot was performed in order to study if the cells are over-expressing the enzymes hGDH1 and hGDH2. In the upper membrane anti-GDH1 antibody was used, while the lower membrane was blotted for GDH2. (G1:GDH1, G2:GDH2,C+:control samples).

On the western plot above we can observe the differences in the expression levels of stable cells compared to the WT. The samples were equally loaded because before the loading of the samples on the gel, it was previously performed bradford assay for the determination of the total protein concentration in the lysate. The comparison on the results above is between the intensity of the signal from stable cells with the WT, which as we can conclude is barely visible.

Our next step was to determine not only the quantity of the GDH expressed in the cells but also if the subcellular localization is as we expected to be and that is inside mitochondria matrix. For the confirmation of that hypothesis, we used co-transfection assay that we had already performed and the results are provided in the fig. 3.10,3.11 showing clearly the localization of the signal coming from mitochondria. In order to confirm these results we decided to perform compartmentation of the cells using a mitochondria isolation protocol from Sigma. By the use of this protocol we managed to obtain from each cell line two fractions,

one containing the mitochondria and the other named cytosolic which practically included the rest of the cell. For the detection of GDHs in the fraction, after the determination of the total protein concentration using Bradford assay, it was performed western blot analysis. The results of the blot are provided bellow in fig. 3.15

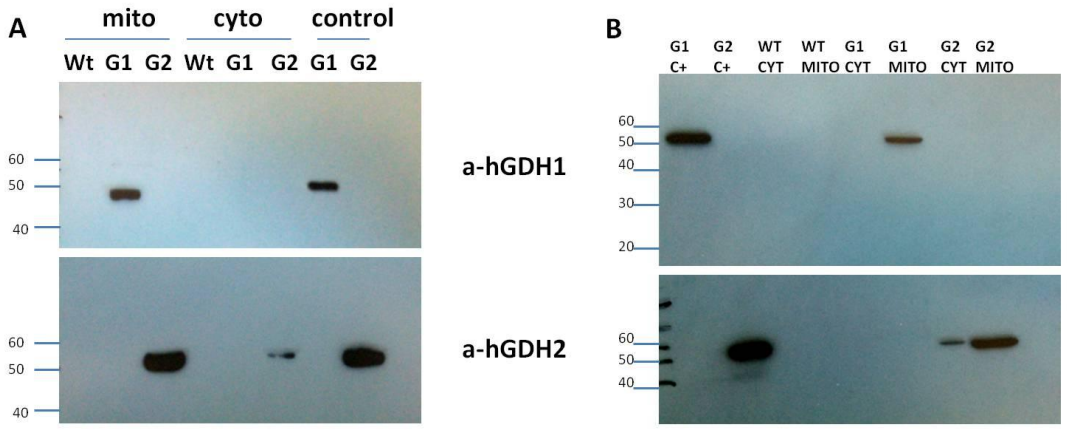

Fig 3.15 In the results provided above we performed western blot analysis of cell lysates after compartmentation of WT and stable cell lines colonies that enabled us to acquire mitochondrial and cytosol fractions. The blot was performed in order to confirm if the cells are over-expressing the enzymes hGDH1 and hGDH2 and whether the enzymes are located in the mitochondria. In the upper membrane anti-GDH1 antibody was used, while the lower membrane was blotted for GDH2. (G1:GDH1, G2:GDH2,C+:control samples).There are two repeats of the same experiment mostly for verification of the results (A and B). A clear band on the mitochondrial fractions of all the stable cell line samples was detected. These results indicate two things. Firstly, the cells are over-expressing hGDH1 & hGDH2. Secondly, they successfully drive the enzymes inside the mitochondrial matrix.

In order to ensure that the isolation of the mitochondria fraction was performed successfully we reprobed our membranes using MnSOD marker which detects mitochondria membrane located protein. The results of the reprobing are in the fig. 3.16.

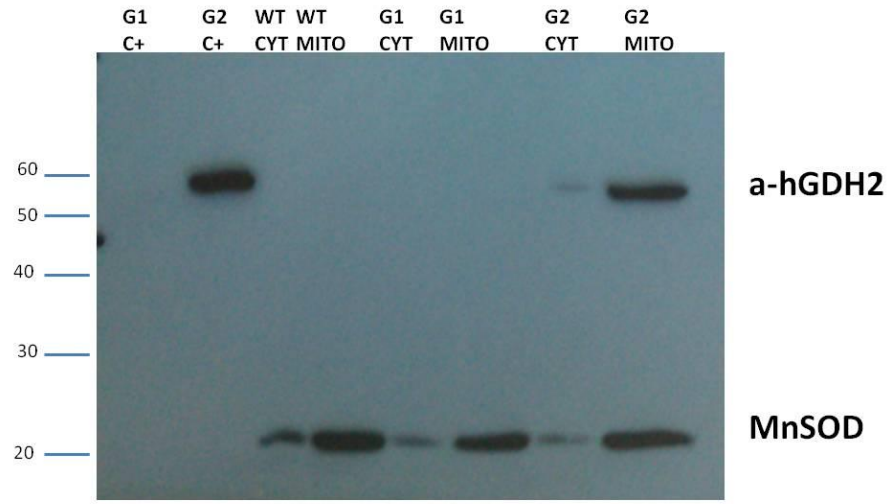

Fig 3.16 Western blot analysis of the wt and stable recombinant HEK 293 cell lines. The antibody used is anti-hGDH2, while reprobing was performed using MnSOD marker. The blot was performed in order to confirm if the compartmentation protocol, for obtaining mitochondria and cytosol fractions was successfully fulfilled. As we can see there is much stronger signal from MnSOD in the mitochondria fraction samples. (G1:GDH1, G2:GDH2,C+:control samples)

SPECTOPHOTOMENTRY-ENZYMATIC ACTIVITY

The last way used for the verification of the over expression of GDHs in HEK 293 cells used was measurement of enzymatic specific activity using a spectrophotometer. From the lysate of the cell line samples we created in vitro reaction and measured the activity of the enzymes. This technique can provide us important information about the performance of the enzymes as well as the quantity of them in the stable cell lines compared to the WT HEK293. The results are provided in the fig 3.17.

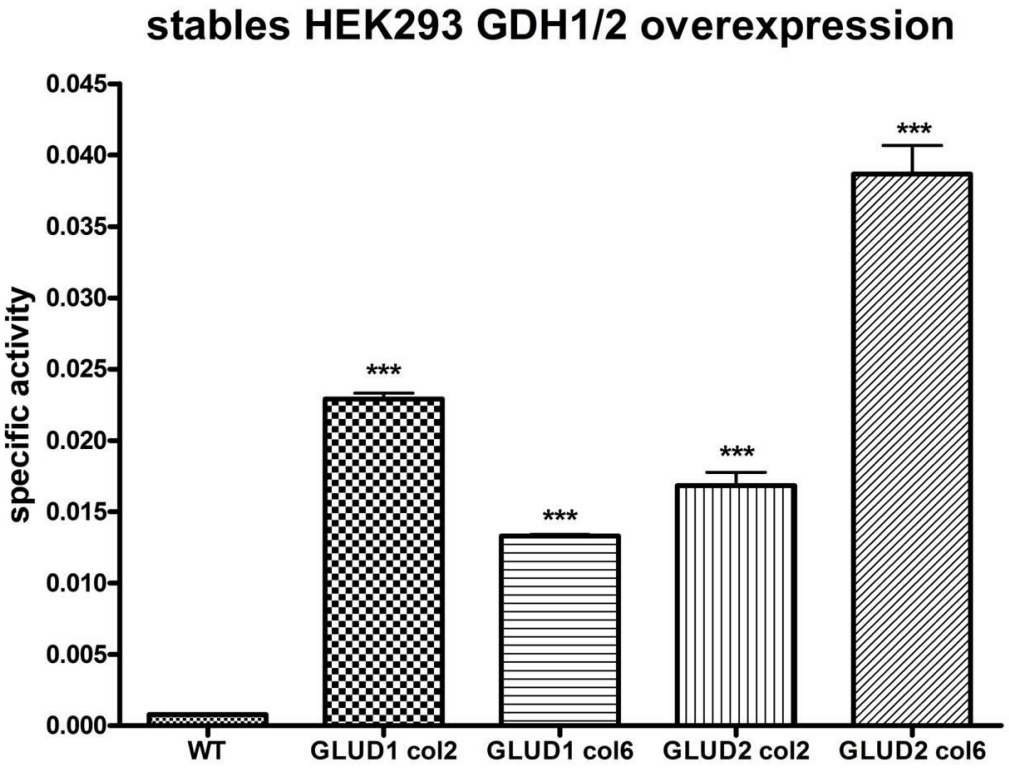

Fig 3.17: Schematic representation of the measurements of the GDHs specific activity achieved using spectrophotometer in a whole cell lysates. The samples used were WT HEK293 (lane 1), stable cell lines over expressing hGDH1 (lanes 2,3) and stable cell lines over expressing hGDH2 (lanes 4,5). The specific activity seems to be notably enhanced in stable cell lines compared to the WT(one way ANOVA, $p<0,0001$).

CHARACTERIZATION EXPERIMENTS ON HEK293 STABLE CELLS FOR THE DETERMINATION OF PHENOTYPIC DIFFERENCES

After fulfilling the goal of providing evidence for the stable cell lines of HEK293 that we created are over expressing hGDH1 and hGDH2, the next target was the determination of the differences stable cells might demonstrate compared to the WT. That project can be supported with evidence in multiple ways.

The starting plan for the study of stable cell lines phenotypic characteristics manifestations was the measurement of growth and death or apoptosis rate changes.

That could be achieved by the help of Trypan Blue staining assay. The blue staining can mark the dead cells in a culture by penetrating their membrane and entering in the cytosol easily. On the other hand, this is something that cannot happen in living cells. By staining the death cells we were able to distinguish their population and measure the living to dead ratio using a hemocytometer under the microscope.

In the fig 3.18 bellow we can see the results from measurements of dead cells present in our cultures in HEK293 stable cell lines at different time points.

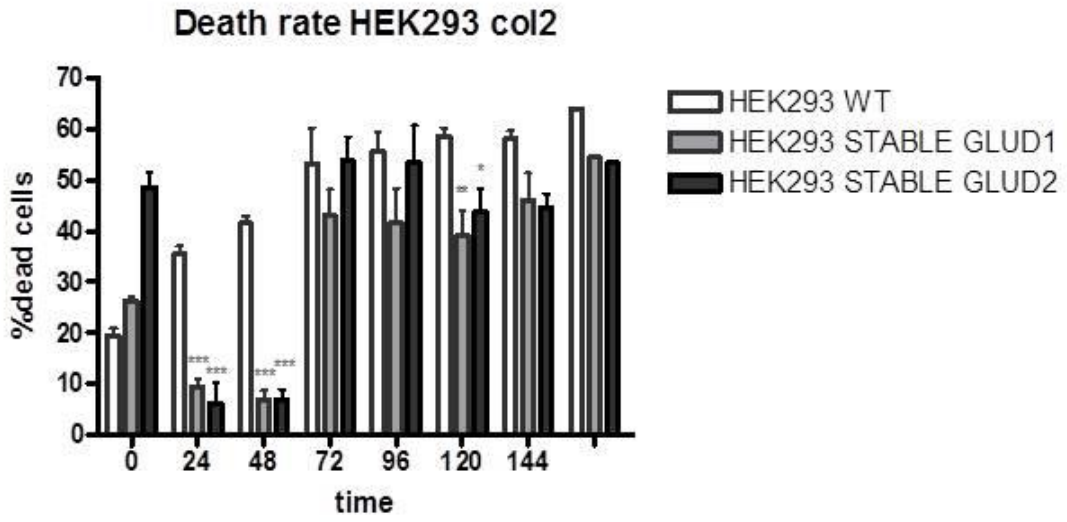

Fig 3.18 Schematic representation of the results from the death rate measurements of stable cell lines over expressing hGDH1 (colony 2, gray lane) and hGDH2 (colony 2, black lane) compared to the WT HEK293 (white lane). Y axis presents percentage of dead cells detected. X axis presents time in hours after plating. The results summarize seven replications of the same experiment. Two-way ANOVA was performed. Bonferroni's post hoc test results are shown in the chart. The biggest differences are observed in time points of 24, 48, 120 hours post plating (* $p<0.05$;** $p<0.01$; *** $p<0.001$)

As observed in the figure above, the results show statistic significance in all time points, even though on time points of 24 and 48 hours the difference on the death rate is more obvious.

The same experiment was repeated on the other two colonies (col6) and the results are provided in the figure following.

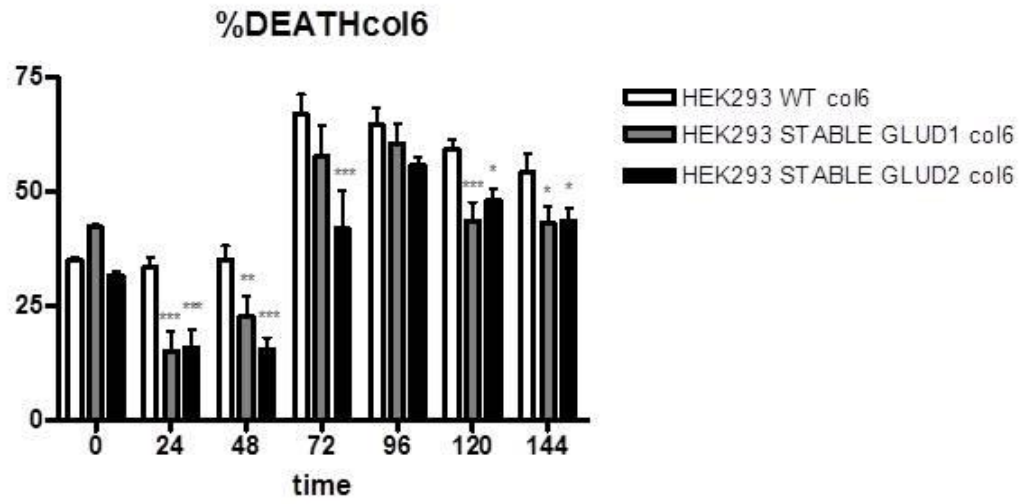

Fig 3.19 Schematic representation of the results from the death rate measurements of stable cell lines over-expressing hGDH1 (colony 6, gray lane) and hGDH2 (colony 6, black lane) compared to the WT HEK293 (white lane). Y axis presents percentage of dead cells detected. X axis presents time in hours after plating. The results summarize six replications of the same experiment. Two-way ANOVA was performed. Bonferroni's post-hoc test results are shown in the chart. The biggest differences are observed in time points of 24, 48, 72, 120 hours post plating (* $p<0.05$;** $p<0.01$; *** $p<0.001$)

The results from all the colonies of stable cell lines are summarized in the figures above. From statistical analysis, we saw that stable HEK293 cells that over express either hGDH1 or hGDH2 show significant differences in death rate. The differences in the number of death cells calculated are surprisingly more intense in all colonies at time points 24 and 48 hours after plating. At the rest of the time points, the difference between stable cell lines and WT cells seems to be minimized. Nevertheless, our statistical analysis revealed statistical significant difference between the stable colonies from WT HEK293 in the total life span of our experiments.

DNA FRAGMENTATION

In Fig 3.20 below we provide the results from the DNA fragmentation experiments. This technique has been reported to detect major apoptotic tendency by taking advantage of the fragmentation occurring on the genomic DNA of the apoptotic cells. In each time point we proceeded in genomic DNA purification using a protocol from thermo scientific. After the genomic DNA from each time point was obtained, the samples were loaded in 1% agarose gel (70V for ~3 hours) and visualized in UV lamp.

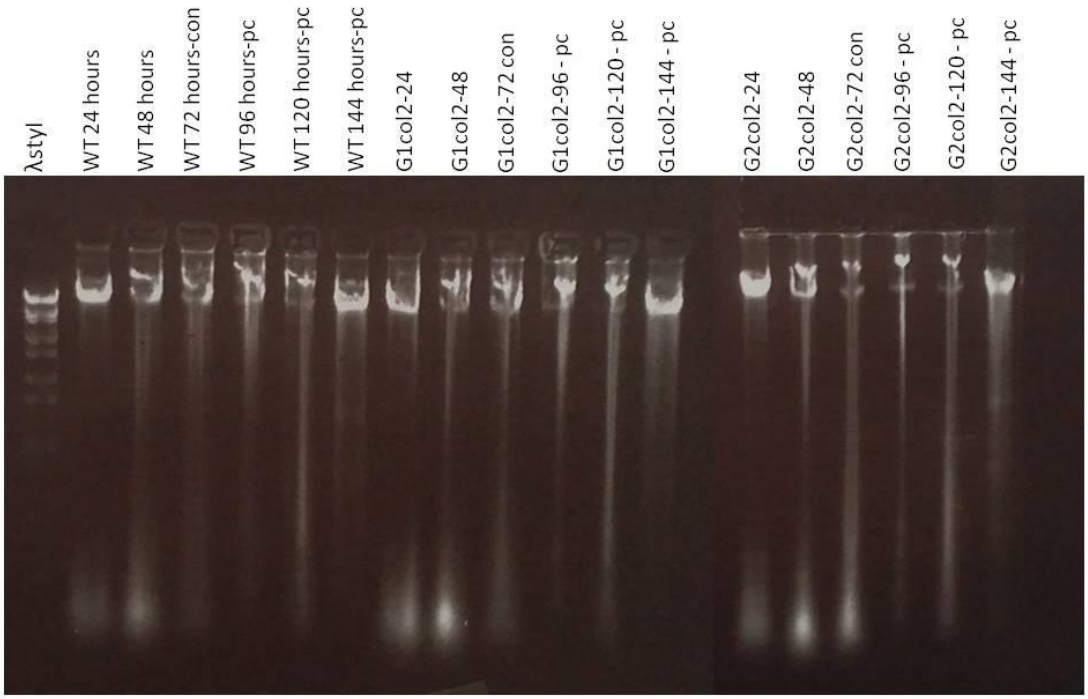

Fig 3.20. Photo of an agarose gel obtained from DNA fragmentation assay using purified genomic DNA from WT, stable HEK293 *GLUD1*, stable HEK293 GLUD2 in different time points. Numbers represent hours after culture. There is no evidence of apoptotic fragmentation in these samples. In time point of 144 hours after plating we are able to observe a sign of fragmentation in WT HEK293 (lane 7) and stable cell line over expressing hGDH2 (last lane) (con: confluency, pc: post confluency)

From the results shown in the gel above (fig. 3.20) we can suggest that there is no intense apoptotic tendency detected in the time points we measured in our experiments. We can roughly support that the over expression of our enzymes does

not seem to cause apoptosis in the cells. Nevertheless, it is wise to note that more experiments investigating the presence of apoptosis in our cell lines need to be performed, for verification of our results.

4. DISCUSSION

In the present study, we provide evidence regarding the answer on two important research inquiries. Specifically, the first part concerns the determination of the subcellular co-localization of the two hGDH iso-enzymes and the second the study of the phenotypic characteristics of stable cell lines that over express hGDH1 or hGDH2. Our results showed that concerning the first part of our study, the isoenzymes do co-localize subcellularly, as detected by the use of confocal microscopy. On the second part of our study we were able to detect reduced percentage of cell death in cultures of stable lines HEK293 over expressing hGDH1 and hGDH2 compared to the WT.

CO-LOCALIZATION EXPERIMENTS

The project about the detection of the subcellular localization of the two enzymes was inspired by previous work of Mastorodemos V. et al, 2009. In this research paper the authors provide evidence for the hGDH1 and hGDH2 that are localized in mitochondria and endoplasmic reticulum. They achieved these results using two construct plasmids, one for each enzyme, expressing chimeric hGDH with GFP. They further verified their results using western blot analysis.

In our project we tried to study if the two enzymes co-localize subcellularly in all the different compartments of mammalian cells. For achieving that target, we needed different staining of each enzyme. For proceeding, we used one construct from Mastorodemos V. work, namely the *GLUD1*-pEGFP plasmid, but the second plasmid with *GLUD2* gene needed to be constructed. For that reason, we managed to generate a construct chimericaly expressing hGDH2 with a RFP tag, using the plasmid mCherry as vector. In the final product of this construct a point mutation was detected from the sequencing, possibly developed by the Taq polymerase enzyme, selected to perform the PCR for obtaining the insert of the gene *GLUD2*. We agreed that this point mutation should have low significance for the experiments we intended to perform since theoretically, it was not affecting the leader peptide that leads the enzymes in specific subcellular locations. Additionally, we proceeded in reconstruction of the same product from the beginning using a high fidelity polymerase this time, for better product and verification of our results. This

construct was verified with sequencing, but was not used to the present study due to time limitations.

After expressing the two constructs in mammalian cell lines, we obtained the results provided in the results section (fig. 3.10, 3.11), showing clearly the co-localization of the two enzymes in mitochondria. Although, in the pictures obtained from SP2 confocal microscope, we could occasionally detect inclusions diffused on our slides expressing high levels of both enzymes but not localized inside cells. We can interpret that the inclusion findings were caused by the over expression of the two enzymes, necessary for our experiments. Another unexpected finding was a diffuse signal of hGDH1-GFP expression in the cytosol, detected in a low number of mammalian cells. The interpretation of that finding could be due to inability of the leader peptide in that case to successfully target the enzyme inside mitochondria. That could happen due to random mutations on these copies of plasmid in those cells or trouble in the correct expression of the protein.

Despite those findings, in the majority of our cells used we were able to detect a complete merge of the signal, proving that hGDH1 and hGDH2 co-localize inside mitochondria. For more detailed localization of the enzymes in compartments of the cell, a more advanced microscope is needed, for improvement of the images captured. Beside the microscope's amelioration, the chimeric enzymes could be used in the future for expression in additional human cell types for confirmation of our findings. Further research could take place in order to verify if they do co-localize in all or in some cell types.

OVER EXPRESSION OF hGDHs EXPERIMENTS

For the second part of our study, our goal is trying to determine which are the phenotypic characteristics that the over expression of both hGDHs could cause in HEK293 cells. The first step for addressing that research goal was to create cell lines that could stably over express hGDH's. The results from our experiments showed that this step was successfully performed and that we managed to create four colonies, two for each hGDH, over expressing many times more enzyme than WT HEK293.

After creating the stable cell lines, our next step was to determine the optimal research ways to study the effect of their overexpression. As we already

know, these isoenzymes play crucial roles in neurotransmittion in CNS, cellular metabolism and in some cases in cell signaling in energy-addicted cancers, according to recent findings (Csibi A. et al., 2013). All these possible functions of hGDHs make it difficult to estimate the possible phenotype of the over expressing cells.

To begin with the phenotypic characterisatoon, it is important to review in detail hGDH's functions. A possible matter to be discussed is if over expression of our enzymes in cells is translated to an over activity. In most of the cases in biology it usually is. This can be explained easily by enzymatic activity measures that show higher enzyme concentration in the reaction, meaning better result in less time (Mastorodemos V. et al., 2005). Does this phenomenon measured in vitro is also valid in vivo were things are for sure more complicated? A possible guess is that the enzymatic activity in vivo should be significantly enhanced due to higher concentration. On the other hand, what one should keep in mind is that hGDHs are equipped with "sensors" that can alter their activity and function allosterically. That might happen because the role of the enzymes in a living cell is defined by the needs of the cell. A part of the significant role of the enzymes inside the human organism is that they can be flexible and adoptable for better service of the cell metabolic demands. As already mentioned in the introduction, the enzyme's activity depends on the GTP-ATP/GDP-ADP ratio inside the cell. Also it is regulated by the presence of leucine or even temperature and PH (Mastorodemos V. et al., 2005, Zaganas I. et al., 2013, 2014, Kanavouras K., et al 2007). Finally, we have already mentioned the functional differences represented between hGDH1 and hGDH2, related to their response in all these factors.

A safe way to begin our study was to measure the stable cell's death or growth rate. That was selected because of the direct relationship of both enzymes with metabolism and consequently life span. The outcome from these experiments revealed differences in the death rate between stable and WT HEK293 cell lines. In our experiments, in certain time points the stable cell lines demonstrate less death cells. There were no statistical significant differences between stable cell lines over expressing hGDH1 with those expressing hGDH2. The differences in death rate between stable and WT cells were higher on the time points 24, 48 and 120 hours in

all of our experiments. Nevertheless, there was a statistically proven difference between all colonies compared to the WT cells.

These results might suggest longer life span of the stable cells. The cells, as we observed in our experiments, are in exponential phase of growth up to 72 hours and in this phase we measured less percentage of dead cells compared to WT line. After 72hours in culture, we can see that our dead cell population is equated until the point of 120hours in which we can once more measure a statistical difference in our results. Further experiments need to be performed for concluding a reliable outcome.

In order to aptly interpret these findings, we need to bend over the biochemical mechanisms of action of hGDHs. In metabolism for example, hGDHs catalyze the reversible deamination of L-glutamate to a-ketoglutarate and ammonia. As we already know the reaction is reversible which practically means that we can't really be aware of the reaction's direction each moment inside a living cell. For addressing that problem we should probably perform signaling experiments. That way we might enlighten what differences in biochemical pathways can be caused by the over expression of hGDHs. Does it fuel more energetically the Krebs cycle by providing a-ketoglutarate and excess of ammonia? Is this over production enough to cause hypoglycemia or hyperammonia inside the cells or is it inhibited effectively by ATP/GTP? If the reaction leans the way of the overproduction of glutamate, can this phenomenon cause excitotoxicity or not? If the production of glutamate is in excess, is it regulated by the help of glutamate synthetase and transformed to non toxic glutamine? There are a lot of questions that need to be addressed in future experiments.

If we review the resent literature, we can detect similar research studies that show over expressed *GLUD1* in neural cells. There are two studies suggesting that over expression of GDH1 in neural cells leads at least to enhanced glutamate release and excitotoxicity. Even though, both studies mention that the biochemical mechanism is not clear yet and that further studies need to be performed (Bao X. et al., 2009, Michaelis E. et al., 2011).

In order to successfully characterize the phenotype of the stable cell lines, a series of experiments need to be performed. We could subject the cells in different

environmental conditions such as serum free or low glutamine or other amino acids LB medium and measure their death rate. Furthermore, we could alter the PH using acidic LB medium or by incubating the cells without providing CO2. That way we can measure if they might represent a sensitivity or resistance compared to the WT cells. Other future experiments could include assays for apoptosis vs death differentiation. Some out of many appropriate techniques for the measurement of apoptosis are the tunnel assay apoptosis kit, or use of an antibody for quantification of caspase 3. Fluorescence-activated cell sorting (FACS) could also be off assistance in this assays

Another important future perspective is also the generation of other human cell lines over expressing hGDHs. That could give as the opportunity to study the various role of the two enzymes in different cell populations.

All these future goals show us that phenotypic characterization of stable cell lines is an achievement that could be very complicated and multifactorial. On the other hand this kind of experiments could provide us with important information about the role of the hGDHs in human organism and specific cell populations. This is of great importance for the understanding of the relation of these enzymes with neurodegenerative diseases and could reveal a possible target for overcoming the excitotoxicity or metabolic failure observed in some of these diseases.

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