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Epigenetic Regulation By The Use Of dCA9-Demethylase In Diabetic Models

- Naif Alhejaili, (Medical Lab Specialist). Email: n.s.alhejaili@gmail.com

- Dr. Colombo, Sergio, (Senior Lecturer). Email: sergio.colombo@ntu.ac.uk

ABSTRACT

This research was aimed at using a genetic tool designed on a dCas9 gene to control the epigenetics of IDH2 in the hydroxymethylation of its promoter region. For the identification of the sequences of sgRNA, cloning and validation of specific sequences for IDH2 was required. Having achieved this, the demethylation of the IDH2 gene was carried out by the use of the modified CRISPR-CAS9 system. Tissue culture transfection of the INS-1 cells with the Dcas9-TET plasmid system was also carried out alongside cell evaluation and DNA isolation. For the amplification and the evaluation of 5-HMC in IDH-2, a qPCR was carried out. Western blotting was the final method used in the verification of the expression of the modified gene expression. The result obtained from this study showed the mechanisms involved in epigenetics which may also apply to a range of diseases including Diabetes associated with mutations in the IDH-2 gene.

Keywords: heredity, reproduction, cells.

ملخص البحث

يهدف هذا البحث إلى استخدام أداة وراثية مصممة على جين dCas9 للتحكم في التخلق المتوالي لـ IDH2 في hydroxymethylationفي منطقة المروج الخاصة به. لتحديد تسلسل sgRNA ، كان الاستنساخ والتحقق من تسلسل محدد لـ IDH2 مطلوبًا. بعد تحقيق ذلك ، تم إجراء إزالة الميثيل من جين IDH2 باستخدام نظام CRISPR-CAS9 المعدل. كما تم إجراء تعداء زراعة الأنسجة لخلايا INS-1 باستخدام نظام البلازميد Dcas9-TET جنبًا إلى جنب مع تقييم الخلية وعزل الحمض النووي.

لتضخيم وتقييم 5 IDH-2في-HMC ، تم إجراء qPCR. النشاف الغربي هو الطريقة الأخيرة المستخدمة في التحقق من التعبير الجيني المعدل. أظهرت النتائج التي تم الحصول عليها من هذه الدراسة الآليات التي ينطوي عليها علم التخلق والتي قد تنطبق أيضًا على مجموعة من الأمراض بما في ذلك مرض السكري المرتبط بالطفرات في جين.IDH-2

الكلمات المفتاحية : الوراثة ، الإستنساخ ،الخلايا .

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INTRODUCTION

Epigenetics

In recent years, researches increased regarding the role of the epigenome in the evolution of various metabolic diseases which includes diabetes; and which involves the changes to the structure and function of chromatin without changing the sequence of DNA (Rosen et al., 2018; Bintu et al., 2016; Golbabapour, Abdulla and Hajrezaei, 2011). Epigenetics, is defined as the study of heritable changes in the expression of genes (active versus inactive genes) this does not involve changes to the underlying DNA sequence (phenotypic change without genotypic change). (Johnson et al., 2015). There are certain important factors that influence epigenetic modifications, namely; lifestyle, environment, individual's age, physical health. These modifications can be divided into two categories – Benign epigenetic modifications which involves the differentiation of cells into specific tissue or organ cells and Harmful epigenetic modifications in which changes are the major cause in the pathogenesis of the adverse health condition such as diabetes mellitus, neurodegenerative diseases and cancer. The physical structure of DNA is modified as response to epigenetic changes, The figure 1 shows DNA methylation, the enrichment of the 5-position carbon in DNA cytosines is due to the methyl group from S-adenosyl-I-methionine (SAM).In addition, the figure 2 shows methylation which involves the enrichment of the 5-position carbon in DNA cytosines with a product; 5-methylcytosine and demethylation which requires the removal of a methyl group. This means that the expression of certain genes is inhibited. Demethylation, which involves the removal of a methyl group from a molecule gives rise to the reversal of the outcome of methylation (Hwang et al. 2017).

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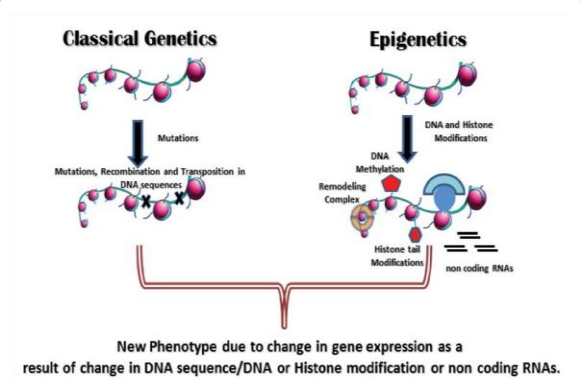

Figure (1) changes in the sequence of DNA, which gives rise to a new phenotype (Yadav,2015).

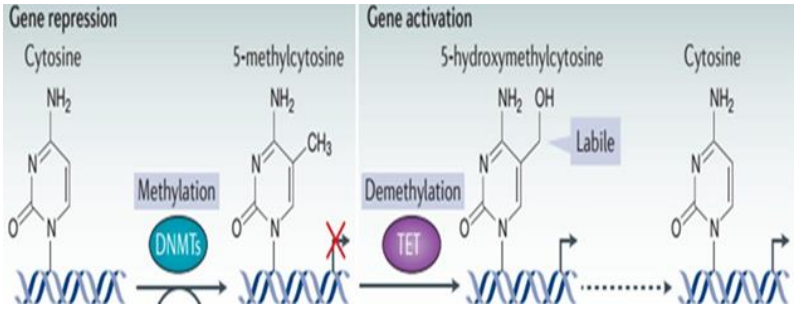

Figure (2): DNA methylation and demethylation (Hwang et al., 2017).

Epigenetic mechanisms

Over the course of an organism's life style, epigenetic changes accumulate, and the next generation inherits these changes if they arise from the germ cells. In a range of multicellular organisms, epigenetic changes facilitate the expression of certain genes, transferring critical data to daughter cells. Researchers have identified a multitude of epigenetic mechanisms including X chromosome inhibition, paramutation, imprinting, gene silencing, modifiable disorder (or phenotypic severity), reprogramming, bookmarking, cancer-inducing processes, cloning maternal characteristics, teratogenic effects and histone change modulation. The Figure 3 Illustrates the primary mechanisms which show the changes in epigenetics.

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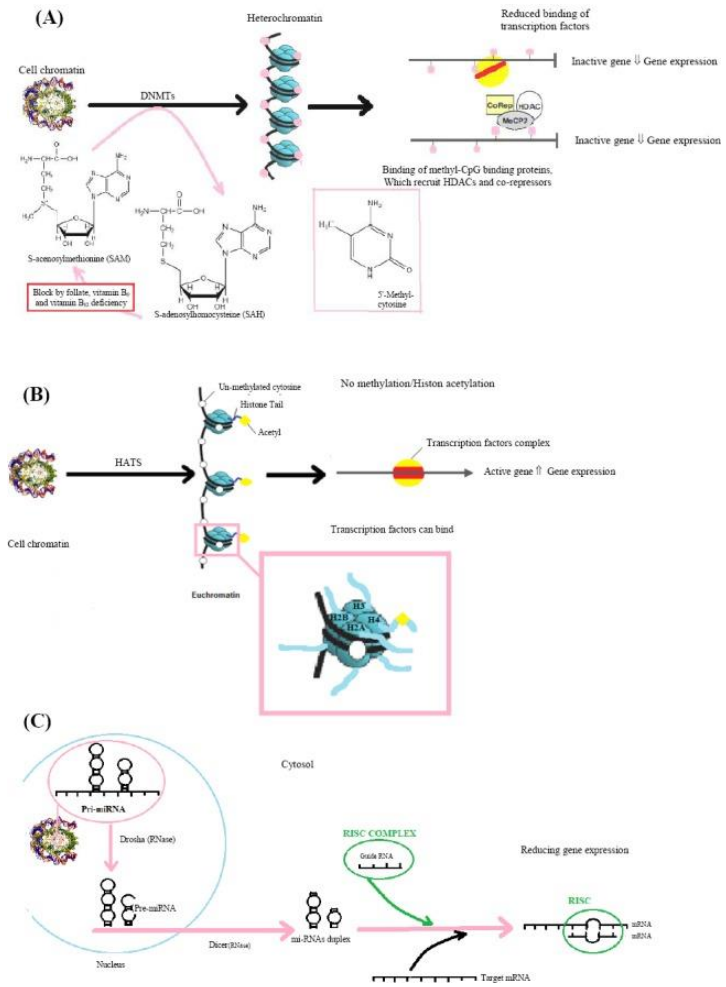

Figure (3): The Mechanisms involved in the modification of epigenetics There are three basic mechanisms involved in epigenetic change; they are (A) DNA methylation, (B) Histone alteration, and (C) Gene silencing, (Moosavi and Ardekani, 2016).

DNA methylation is the primary pathway involved in epigenetic changes. This involves the addition of a methyl group at the nucleotide; cytosine to the fragment of DNA. The primary constituent in epigenetic control of diabetes and its complications through the metabolic memory as shown in Figure 4 (Reddy, Zhang and Natarajan, 2014; Al-Haddad et al., 2016) is Histone modifications, which involves arginine and lysine methylation. This is also the second most important epigenetic mechanism, in addition to acetylation (Rosen et al., 2018).

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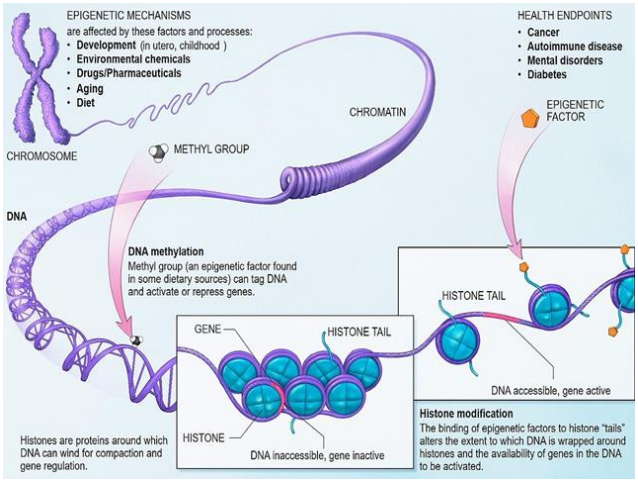
Changes in ncRNAs, including miRNAs and piwi RNAs and also long non-coding RNAs further occur (Keating et al., 2016).



Pul

5-hmC play several roles for T-even phages as shown by several accounts. One perspective considers 5-hmC and its glucosylation to be involved in phage host-controlled restriction, this is an idea which comes from observing that for the uridine diphosphoglucose (UDPG) pyrophosphorylase bacteria, it is impossible to create the glucosyl donor UDPG which is needed for the glucosylation of 5-hmC. The phage undergoes defective glucosylation due to this and therefore not protected from bacteria restriction endonucleases (Hattman, 2009). Wiberg, 1967 also suggests that the functions of both glucosylated and unglucosylated 5-hmC is to protect progeny phage DNA from host nuclease degradation. An Empirical evidence showing that cytosine-containing DNA in mutant phage, characterised by the sensitivity with respect to phage-controlled nucleases supports this view. Kutter and Wiberg, 1969 also considers that 5-hmC and not cytosine-containing DNA is important when the later genes which encodes for phage structural proteins and lysozyme are transcribed.

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dCAS9 system

dCAS9 is a modulator system for transcriptional regulators attachment. It fuses easily with effectors, which are either transcription activators or repressors for the purpose of targeted gene regulation (Baliou et al; 2017), as shown in Figure 6. Mutated nuclease domains of Cas9 from S. pyogens which involved an H840A mutation in the HNH domain and a D10A mutation in the RuvC domain created the deficient dCAS9 (Baliou et al. 2017). The ten-eleven translocation (TET) enzymes especially TET1, TET2 and TET3 play a role in the co-ordination of processes related to demethylation, they convert 5-mC into 5-hmC, in the presence of 2-oxoglutarate and iron (II), this occurs without the involvement of DNMT. A DNA glycosylase able to excise 5-mC occurs through ROS1; this is a direct demethylation pathway. For the pathway of DNA methylation editing to be completed, dCas9 is fused with enzymes in the pathway.An earlier study conducted by Bernstein et al., 2015 targeting particular CpGs through the TALE system showed that TET1 and DNMT3a were the system effectors used.

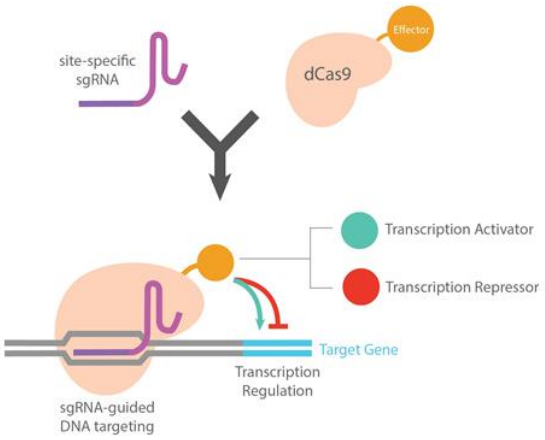

Figure (6): dCAS9 as a modulator system for transcriptional regulators attachment (Baliou et al. 2017).

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Predictors of the risk of developing type 2 diabetes are poor physical fitness and a low Vo2max (Eriksson and Lindgärde, 1996). There exists a link between poor physical fitness and an increased risk for disease, this includes changes in muscle fibre-type composition and insulin resistance. The expression of a number of genes that regulate glucose uptake in skeletal muscle, including GLUT isoform 4 (GLUT4) can be induced by exercise. *Furthermore*, Aging has an association with an increase in the risk of type 2 diabetes. Oxidative capacity and mitochondrial function reduce with aging and also in patients with type 2 diabetes (Ritov et al., 2005; Petersen et al., 2003). This may be due to genetic and environmental mechanisms (Bua et al.,2006). Suggestions from recent data show that epigenetic pattern may be transformed as life progresses, this affecting the key genes involved in the respiratory chain (Fraga et al.,2005; Rönn et al.,2008).The data gathered shows the impact of aging on DNA methylation, gene expression and also, in vivo metabolism. Genetic factors may further be an influence on the interaction between mon-genetic and epigenetic mechanisms. Another important consideration in aging and type 2 diabetes is Hepatic insulin resistance. The key enzyme in hepatic glucose utilization is Glucokinase however, its activity is reduced in the liver of diabetics. This may be due to mutations in the glucokinase gene, which causes a monogenic form of diabetes called maturity-onset diabetes of the young (MODY) (Yang and Chan, 2016). It has also been discovered that although aging is associated with gene-specific hypermethylation, many mammalian tissues show global DNA hypomethylation and a reduced methyltransferase (DNMT1 and DNMT3a) expression with an increase in age (Ling et al., 2004; Rönn et al., 2008; Jiang et al., 2008). The repetitive sequences are an evidence of global hypomethylation, this promotes genomic instability during aging.

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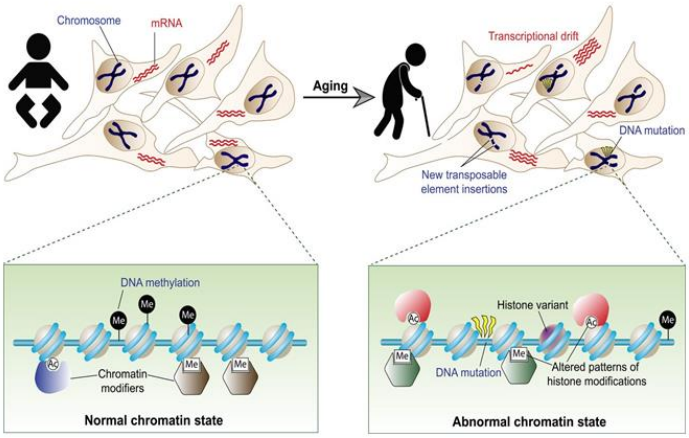

Figure (7) : An overview of epigenetic changes in aging (Pal and Tyler, 2016)

The relationships between maternal obesity, diabetes and nutrition in the course of pregnancy with birth weight and also relationships between birth weight and the risk of obesity and diabetes at later stages of life have been observed (Gluckman et al., 2008). One major factor that plays a role in this is epigenetics. Epigenetic research into diabetes, especially type 2 diabetes (T2D) is still in its early phase. Epigenetic changes of T2D coveys the interaction between the activation of genes and epidemiology, here gene activation can be through DNA methylation, histone modification or RNA activation or also through different epidemiological factors such as age, obesity, nutrition, physically active and intrauterine environment (Davegårdh et al., 2018). Histone acetyl transferases (HATs) and HDACs majorly regulate the genes linked to diabetes. HATs play a role in the expression of genes in endothelial cells, this is a process initiated by glucose.

Genomic imprinting is another epigenetic phenomenon that is well documented in humans, occurring during germ cell development with the regulatory regions of certain genes methylated differently and expressed depending on inheritance from either father or mother (Kubota, Miyake and Hirasawa, 2012). During imprinting, several genes are affected, this may lead to the occurrence of mutation of the expressed copy or the disturbance of normal imprinting; this is seen mostly in the rare transient neonatal form of diabetes and also in some cases of polygenic type 1 and 2 diabetes (Pollin, 2011).

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Oxidative stress plays a major role in the development of complications of diabetes, both cardiovascular and microvascular. Metabolic abnormalities involved in diabetes lead to mitochondrial superoxide overproduction in endothelial cells of both large and small vessels, and also in the myocardium. There are five (5) known pathways involved in the progression of diabetes complications namely: polyol pathway flux, increased formation of AGEs (advanced glycation end-products), increased expression of the receptors for AGEs and its activating ligands, protein kinase C isoforms activation and overactivity of the hexosamine pathway. Each of these pathways leads to different complications (Villeneuve, Reddy andNatarajan, 2012)

The pathophysiology has been connected to the upregulation of a number of growth factors and lipids that trigger most of the signalling pathways, activation of transcription factors and then epigenetic interaction. When epigenetic alterations persist, metabolic memory is induced. This has been suggested as also inducing an increased risk for diabetic complications. (Zheng et al., 2017)

Mutation in genes encoding transcription factors which may include HNF1A, -4A, -1B, IPF1/PDX1 and NEUROD1, which may regulate transcription of their target genes through associations with HAT and HDACs cause most forms of maturity-onset diabetes of the young (MODY) as shown in Figure 8. Transcription is activated by HNF1α activities through two different mechanisms, namely – the general transcription machinery recruitment and chromatin remodelling of promoter regions (Párrizas et al., 2001). HATs (p300/CBP) are recruited in the remodelling of chromatin, this causes a hyperacetylation of histones in β-cells at specific promoters including GLUT2 and pyruvate kinase. A missense mutation (R263L) in the HNF1A gene associated with MODY phenotype leads to a decreased affinity for p300 (Kim et al., 2003). The ability of the HNF1β to bind proteins with HAT activity is influenced by the MODY mutations in the HNF1B gene, this then affects the chromatin structure (Barbacci et al., 2004).

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Moreover, Pdx1 regulates glucose-induced expression of insulin in β-cells, this needs an interaction between Pdx1 and p300, followed by a hyperacetylation of histone H4 at the insulin gene promoter (Qiu et al., 2002; Stanojevic, Habener and Thomas, 2004). A low level of glucose decreases the expression of insulin expression due to the recruitment of HDAC1 and HDAC2 by Pdx1 (Mosley and Ozcan, 2004).

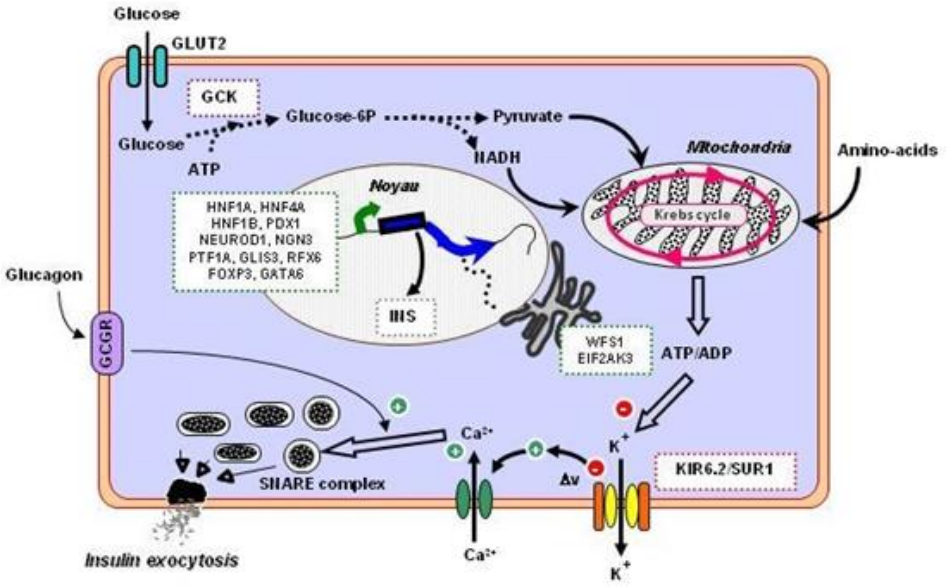

Figure (8): Monogenic diabetes and the epigenetic factors involved (Murphy, Ellard and Hattersley, 2008).

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IDH2 and diabetes (The role of IDH2 in insulin secretion and dysfunction of the mitochondria)

The Human Isocitrate dehydrogenase (IDH) has three isozymes, namely – IDH1, IDH2 and IDH3. IDH1 is found in the cytoplasm while IDH2 and IDH3 are found in the mitochondria. IDH is involved in the tricarboxylic acid (TCA) cycle, and it catalyzes the reversible conversion of isocitrate to alpha-ketoglutarate (α-KG)/2-oxoglutarate (2-OG), further promoting the dioxygenase activity requiring α-KG as a cosubstrate, NADP+ is used as the cofactor for IDH1 and IDH2, it produces NADPH which plays a vital role in the regeneration of the antioxidant glutathione. IDH3 uses the cofactor NAD+, producing NADH. IDH2 plays a vital role in reactive oxygen species (ROS) which are by products of normal metabolism with a role in cell signalling and homeostasis. The mitochondria are the main organelle involved in intracellular ROS production, its role is in the modulation of redox- dependent processes in cells namely; metabolism and apoptosis. IDH2 further helps in the prevention of age-related diseases (White et al., 2018). IDH2 is also important in cell proliferation. In several proteomic studies, several possible lysine acetylation and acetylation sites have been observed in IDH2 (Xu et al., 2017). In one of the proteomic studies, Lys106 and Midh2 (mouse mitochondrial IDH2) were identified as a lysine acetylation site in two Lys 272 samples (fed and fasted) (Sol et al., 2012).

A study carried out by Lee et al., 2016 showed that mice with IDH2 disruption showed resistance to obesity, had lower body weight, reduced visceral fat, increase in sensitivity to insulin and high energy expenditure relative to controls. This is linked to the ability of IDH2 to suppress lipogenesis in visceral adipose tissue, with an increase in thermogenesis in the adipocytes by transcriptional activation of UCP1 through the activation of the p38 signalling axis. Lipogenesis is suppressed through the transcriptional repression of SREBP1. The importance of a redox balance in metabolism regulation and the role of IDH2 in modulating insulin sensitivity and fuel metabolism is highlighted in the result of the study. This leads to the establishment of the protein as a possible target in type 2 diabetes and obesity treatment.

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AIMS

The key aim of this study was to use a genetic tool designed on a dCas9 gene to manipulate the epigenetics of IDH2 in the hydroxymethylation of its promoter region. In addition, the following sub- objectives:

- identify specific guidance RNA sequences.
- clone these in a pLentiPuro-gRNA vector.
- validate the sequence and the amplification of resulting plasmids.
- carry out cell transfection with INS-1 cells.
- quantify the effect of hydroxymethylation in IDH2 by qPCR after treating with this tool.
- functionally validate the tool by assessing epigenetic changes on the IDH2 gene by western blot.

MATERIALS AND METHODS

FIRST: Cloning of guidance INS sequences

1. Plasmid DNA purification:

Materials: Centrifuge,Buffers (P1, P2, BB, S3), QIAfilter Cartridge, QIAGEN plasmid plus spin, Buffers (ETR, PE, EB), 1.5 ml tube, Pipettes and Tips and NanoDrop machine (to measure the DNA concentration).

Experiment Procedure: Bacteria culture was harvested by centrifugation at 6000 x g for 15 minutes at 4°C, pelleted bacteria resuspended in 4 ml Buffer P1. Then, 4 ml Buffer P2 was added and gently mixed by turning upside down until the lysate appeared viscous, incubation followed at room temperature (15-25°C) for 3 minutes. Cell suspension turned blue due to the addition of LyseBlue reagent.

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A new and suitable was used in the placement of the QIAfilter Cartridge, Buffer BB was also placed in the tube, 4 ml Buffer S3 was then added to the lysate and mixed six times by turning upside down. Due to the addition of LyseBlue reagent, the solution was thoroughly mixed until it appeared colorless. After that, transfer the solution to the QIAfilter Cartridge and incubate at room temperature for 10 min. After the incubation 10 min, need to insert the plunger into the QIAfilter Cartridge and filter the cell lysate into the tube. 2 ml Buffer BB was added to the cleared lysate and mixed for 4-6 times by turning upside down. After this step, took 1 ml (800 ul) of the solution to add it to Qiagen plasmid plus spin column and centrifuge 1 min at 1500mbar (need to dispose the liquid after vacuum, and will repeat that step until the solution is completely finished. DNA was washed by the addition of 0.7 ml (700 ul) Buffer ETR and vacuum used until the liquid was drawn through all columns. Then, 0.7 ml (700 ul) buffer PE as well to wash the DNA further wash the DNA. After that, will take the solution to centrifuge without anything, to ensure the DNA is concerted at the end of tube. After that, we took the solution to the centrifuge in order to vacuum it again at 9700 rpm for 1 min without anything, to ensure the DNA is concentrated down the tube. Then, Plasmid Plus spin column was finally placed into a clean 1.5 ml tube. Finally, to elute the DNA, added 200 µl Buffer EB to the centre of the QIAGEN Plasmid Plus column to and it was let to stand for ≥1minute and centrifuged for 1minute. After the final step can measure the concentration of DNA via machine.

2.Sterile agar medium preparation:

This was the procedure used for the E. coli Fast-media preparation, and this is done using LB-based agar medium, which was supplemented with Ampicillin. A sterile growth media was prepared using clean glassware, and the E. coli Fast-media pouch was poured into a 500 ml borosilicate glass bottle with an addition of 200 ml distilled water. The microwave oven was placed at 450 W and the medium heat for 3 minutes until bubbles began to appear. The preparation was gently swirled for proper mixing, and it was then re-heated for 30 seconds and swirled gently again. It was carefully monitored so as not to overboil and be sure it completely dissolved. Colonies in the plate after it was incubated were observed, a colony was then taken and placed in a tube with 4 ml media and left overnight in an incubator, this approach was taken so as to increase colony growth.

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3.Cloning of guidance RNA against IDH2

5 ug of the lentiviral CRISPR plasmid with BsmBl was digested and dephosphorylated for 30 minutes at 37 °C. A 60 ul reaction was prepared by the addition of 3 ul BsmBl, 3 ul FastAP, 6 Ul of 10X fast digest buffer and finally 100 mM DTT (freshly prepared). The digested plasmid was gel purified using QIAquick Gel Extraction kit and elute in EB. Each pair of oligos was phosphorylated and annealed forming a 10 ul reaction by the addition of 1 ul oligo 1 (100 uM), 1 ul oligo 2 (100uM), 1 ul 10X T4, ligation buffer (NEB), and 6.5 ul ddH2O, 0.5 ul T4 PNK (NEB M0201S). The reaction was placed in a thermocycler at 37 °C for 30 minutes and 95 °C for 5 minutes, and then ramped down to 25 °C at 5°C/min. The annealed oligos is diluted at 1:200 dilution into sterile water or EB. And a ligation reaction was set up, incubating at room for 10 minutes. A negative control ligation and transformation was also carried out.

4. Bacterial transformation

NEB® Turbo Competent Escherichia coli (E. coli) was the product of the transformation used for the needed recombinant plasmids. The competent cells kit provided the guidance for the procedure, and this was carried out by using the procedure suggested with the heat shock. Recombinant plasmids were firstly introduced into the competent cells, LB agar plates with ampicillin was used for the plating of the transformed cells. This was carried out for 12hours, the transformed cells were further incubated at 37 °C. 10 colonies were then chosen from each of the agar plates, these were re-cultured using a 15 ml tube, 2 ml of LB broth contained ampicillin. E. coli transformation was carried out to allow plasmid storage and replication without DNA rearrangement. Specific treatments were used for the E. coli bacterial competent cells, and this was done to increase the efficiency of transformation. Only cells with growth and colonies on the agar plates were considered as those with plasmids. A colony was further chosen from every plate and incubated independently with the use of a LB+AMP medium. By using the agar medium, a control plate was created, this helped in creating the differences in the consistency of agar medium after incubation.

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5.Confirmation of cloning by DNA sequencing

Plasmid were sent for sequencing to Source BIOSCIENCE NOTTINGHAM

SECOND: Validation of constructs in tissue culture

1.Cell culture:

INS-1 cells were provided by Dr. Colombo. For culture, this cell the media used was, RPMI supplemented with the following 1% L- Glutamine (stock 200mM), 10% fetal bovine serum and 1% penicillin streptomycin (10,000U/ml). For the generation of stable cell line, treatment with puromycin (1ug/mL) was used, this was followed by incubation with 5% CO2 at 37 °C. The cell line was maintained by sub-culturing at 3-4 days intervals.

2.Cell Transfection

In order to check the functional plasmid generated INS transfected using PEI reagent. Briefly, cells seeded in 6 well plates were used for the transfection of cells with the constructed plasmids, they had the sequences needed for the production of guidance RNA, also with the plasmid encoding the dCAS9-TET sequence. The confluence of cell observed was around 70%. Assessment of the efficiency of transfection was than by fluorene microscopic. Transfected colonies were selected with puromycin as described as before. The complex mixture was then added to different wells in the plate. Between 6-8 points in the six wells, were agitated by gently shaking the plates back and forth. This was repeated three times with cells incubated overnight at 37 °C with CO2.

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THIRED: Assessment of hydroxymethylation

1. DNA extraction

Cell growth was confirmed under a microscope before the media was removed from the 6- well plate, the cells were rinsed with 2 ml PBS at pH 7.4. By the addition of 500 µl of trypsin, cells were removed from the wells to different wells for 5 minutes. To each well, 2 ml of PBS was further added and the mixture from the three wells transferred to a tube labelled with DNA using a pipette. Furthermore, the transfer of the remaining solutions in three wells to another falcon tube labelled with proteins was carried out. The sample was centrifuged at 300 xg for 5 minutes, discarding the supernatant. In 200 µl PBS at pH 7.4, the remaining pellet was left suspended with the addition of 20 µl Proteinase K solution and then further transferred to a micro-centrifuge tube. The second step involved the addition of 200 µl Buffer AL (lysis solution) and thoroughly mixing the solution by vortexing. Incubation at 56 °C for 10 minutes followed, 200 µl ethanol (96%) was further added and mixed by vortexing. The sample was then pipetted into a DNeasy Mini Spin Column 2 ml collection tube and centrifuged at 6500 xg for 1 minute. The spin column was placed in a new 2 ml collection tube, the flow through and collection tube then discarded. 500 µl wash buffer 1 (Buffer AW1) was used in washing the tube, the constituents were 19 ml concentrate and 25 ml ethanol; centrifuged at 6500 xg for 1 minute. This process was again repeated and washed with AW 2, the constituents were 13 ml concentrate and 30 ml ethanol and centrifuged at 20000 xg for 3 minutes. The flow through and collection tube were both discarded, and a 2 ml microcentrifuge tube was used in transferring the spin column. 200 µl of buffer was added to the centre of the spin column membrane, then incubated at 1 minute to eluate the DNA from the tube. This was subsequently centrifuged for 1 minute at 6500 xg and the spin column discarded afterwards. The micro-centrifuge tube was kept for measuring using the qPCR.

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2. 5-hmc determination; Restriction sensitivity assay

This follows after three culture experiments have been carried out for the cell line INS-1. Extraction and measurement of DNA from each of the cell line was carried out under three conditions of nutrients namely glucose, galactose and acetate. Samples were investigated as follows O.3 µg, 1.034 µg and 1.031 µg of cell line INS respectively. Each was incubated overnight at a temperature of 37 °C with dH2O, smart buffer was cut both in the presence and the absence of T4BGT (T4 beta-glucosyltransferase) and UDP-glucose (Uridine diphosphate glucose) and positive glucosylation reaction (samples with T4BGT and UDP-glucose) or negative glucosylation reaction (samples without T4BGT and UDP-glucose). Glucosylation reactions positive or negative for each cell line was 18, with a total volume of 20 ul for the INS cell line single reaction and 30 ul for INS cell line. The incubation was followed by performing both +ve and -ve endonuclease restriction digestion reactions for both positive and negative glucosylation reactions. The positive endonuclease restriction digestion reaction was performed by the addition of the enzyme MSPI in addition to dH2O and CUT SMART buffer while the negative endonuclease restriction digestion reaction was carried out by using dH2O instead of MSPI in both positive and negative glucosylation reactions (The kit from New England Biolabs company provided the MSPI and CUT SMART used). For the +ve and -ve endonuclease restriction digestion reaction samples, the +ve and -ve glucosylation reactions for the cell line DNA was 36. The total volume for each +ve and -ve endonuclease restriction digestion reactions was 50 ul. These were finally incubated at 37°C for two hours.

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Fourthly: Assessment of protein expression

1.Western blot

For the yielding of a qualitative result from a Western Blot analysis, every protein sample must have equal quantity of protein. The succession amount was around 20-50. Using a falcon tube, 1.5 ml of Reagents containing the western ECL substrate peroxide was mixed with ECL developer lumino/enhancer from Bio-Rad. The solution produced was then pipetted onto the membrane to detect the level of antibody reactivity. For the creation of the blot image for analysis ChemiDoc MP system was used, the processing was carried out through the image Lab TM software.

RESULTS AND DISCUSSION

RESULTS

1. Identification of IDH2 promoter region

Applying the gene browser feature in the database of the genome, the analysis of target sequences was carried out. This enabled the putative guide RNA sequences to be identified.Most CRISPR systems validate the Cas9 protein, the Cas9 proteins are the catalyst for breaksof DNA at genome locations. This presents as a match with the 20mer guide segment of the CAS9-bound CRISPR RNA (crRNA).

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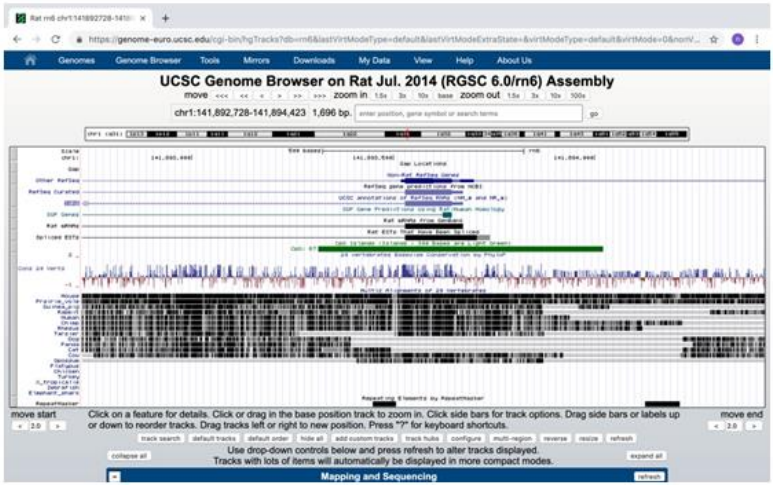

Shows a screen shot highlighting IDH2 gene and CpG islands identification using a software. These are also shown in Green colour (genome.ucsc.edu).

```
>rn6_cpgIslandExt_CpG: 67 range=chr1:141893315-141894022 5'pad=0 3'pad=0 strand=- repeatMasking=none
CGCCTCGGGTCGGTGGGTCAGCTAAACGCACAGGGCGCAAGGGATTAAGG CGACGTTTCCGCCGCTGACATTCTTATAGAAGCGTGAGTCTTGCAGCCAT
AAGCTCCCGCGGCCGATGGGTCTTACTGCTGCTTTGAGGATGTATAGAGG ATCCCAGAAAGCGCGGGCTCAGCGTTGGGGCCGCCCGGGCTCTGGAACAG
GCCCAAGGCTGTCGGGCAGGCTCCTGTCCCGGCCGTGGACCCGCCTTCTC CCGGGTGAGACCCAATGGGGCGGCGGGTTCTGCAGCTCCGCCCCGGTTGG
GTCCCAGCCACCAAGGCCAGCCGAGTCCCCACCGCACAGCCCGGTGCAGC GGCGCGCTCGGACCTCGTGTGCTCGGGATGGCCGGCTATCTGCGGGCCGT
GAGCTCGCTCTGCAGAGCCTCGGGCTCAACACGGACCTGGGCACCGGCTG CACTGAATGTCCCAGCTGGCCGGAGCAGCCGCGGCGCCACTGTGAGTGT
CGCACTGGGGGGACGGGGGGGGGCGCGGCGCGCGCGCAGCGCTGCGAC GTGGCGACGTCCCGGGAGGGGGACCCTGGAGCGTTACTCGCGACGTGCCG
GCGGTCGGGGAACGGCGGCGTGCGGGTTACAAAGGACCGGGAGGTGGCCT TACTCCGGGCTCCCGCGCTTGTGGCTCCTGGGCCGCTGCAGGCGTAGGGA
TGGGGACG
```

Figure (9) : Shows the target sequence identification, this happens near the special CpG islands, they are also usually present in the IDH2 gene promoter region. The CCGG sequence identified is also shown and this highlights the analysis of hydroxymethylation as possible pLPGRNA constructs as the DNA methylase identifies the CpG islands.

2. Prediction for guidance RNA sequences using the software and sequence validation

The E-crisp.org software, which is based on CpG island sequence, targeting the 20-base pair sequence was used. It determined the nucleotide sequence with a score. Snap gene was used in the visualization of the gRNA.

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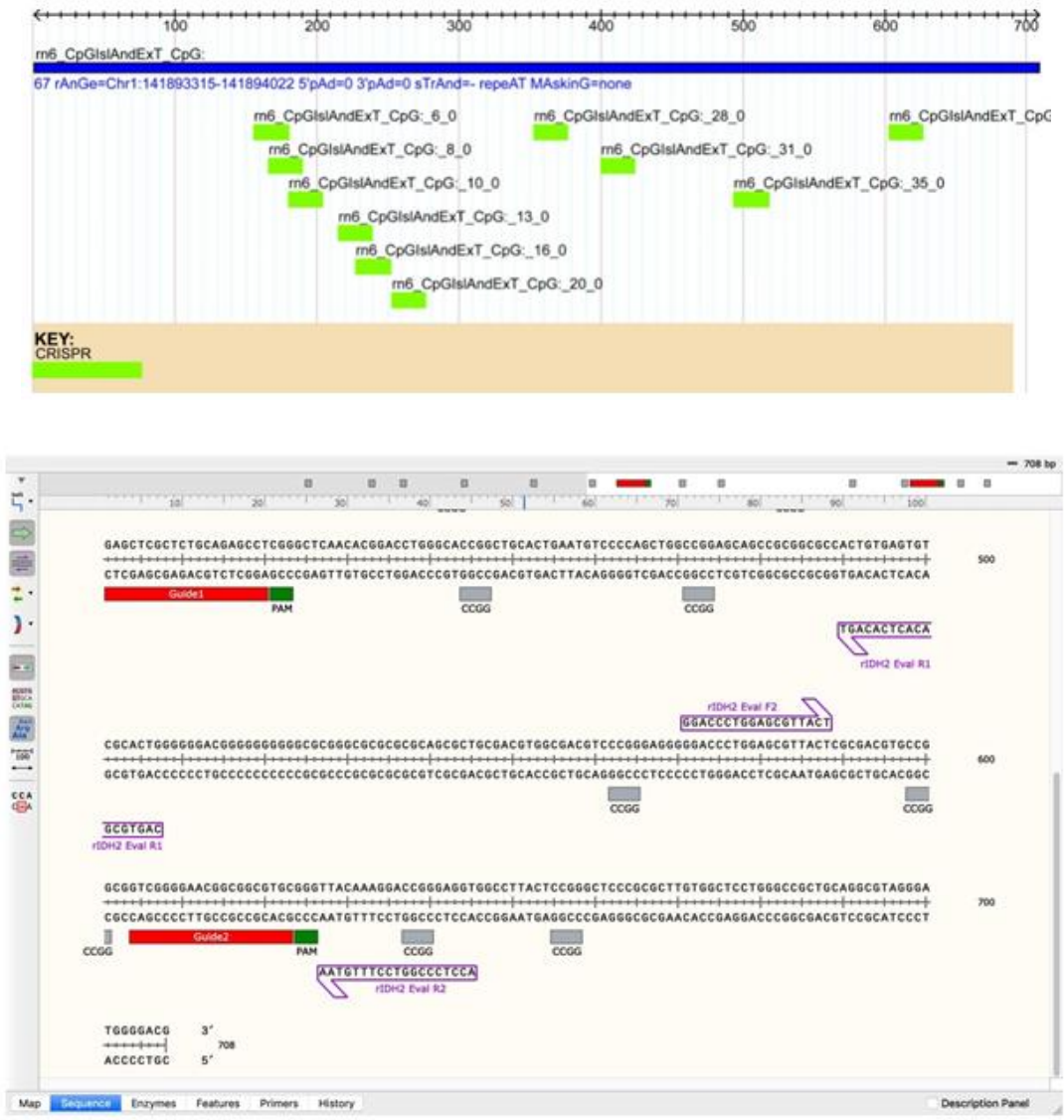

Figure(10) : Shows the IDH2 promoter region and also the primer used for qPCR after clicking the gRNA on the promoter region. Three boxes: Grey, Red and purple are shown. The grey box represents CCGG sequences identification, the red box represents the identified guidance RNA sequence while the specific primers used in the qPCR are represented by the purple box.

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3. Cloning of targeted guidance RNA sequence

Cloning was carried out in the pLentiPuro-guidance vector. This was followed by Sanger sequencing. E. coli was used in the replication of the final plasmid and with the use of the BsmBl enzyme, the pLentiPuro

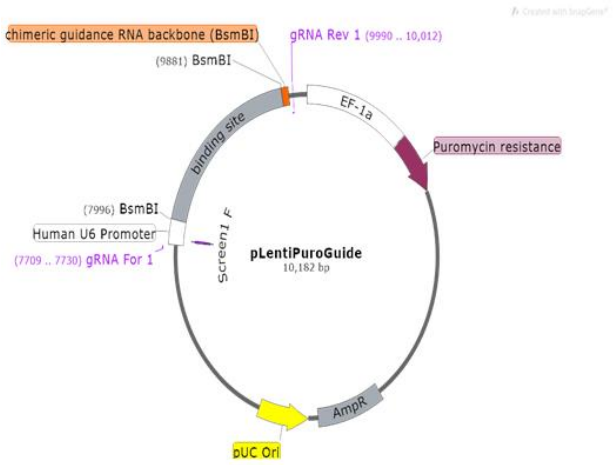

Figure (11): Shows a diagrammatic description of the pLentiPuro Guide vector. Restriction sites of BsmBl and part of the guidance RNA structure are shown using SnapGene

4. Plasmid Amplification and vector preparation

BsmBl; a restriction enzyme was obtained and used to digest gRNA and the recombinant plasmids; Plenti-puro. The E. coli backbone was digested using the restriction enzyme with the insertion of the PCR product. With regards to the primers (forward and reverse), insertion occurred at these two sites. The insertion of PCR products occurred regarding the E. coli backbone that had been digested using the restriction enzymes. The product is the digested vector.

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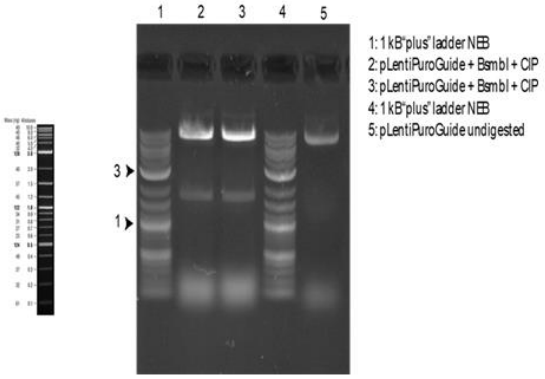

Figure (12): Shows agarose electrophoresis of the digestion of pLentiPuro-gRNA (pLPGRNA) with BsmBl. An agarose electrophoresis (0.8% agarose, refer to material and methods for further details) was used in the separation of the products

5. Bacteria Growth features and positive clones

The expected plasmid showed positive clones. Colonies which surfaces are circular, smooth and white coloured grew in the agar plate.

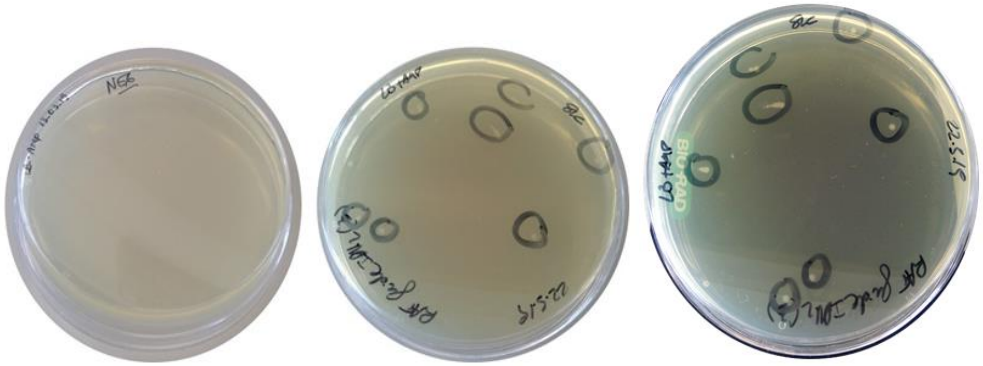

Figure (13): Selection of positive clones of E. coli carrying the pGuideRNA IDH2. Positive colonies were selected on ampicillin plates. Surfaces possessing a circular, smooth and white pigmented colonies produced 150 µl of IDH-2. Some of the cells grew, divided and formed colonies in the agar plate.

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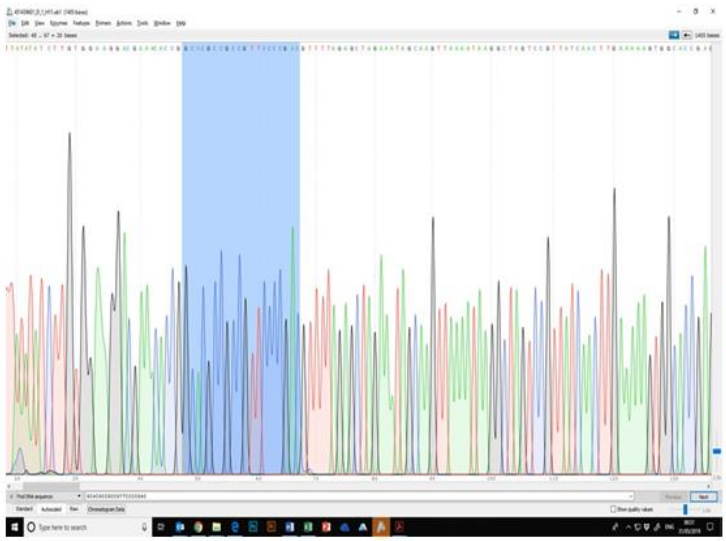

Figure (14): Shows the confirmatory sequencing, guidance RNA for RAT IDH2 promoter region. The main goal of validation is to determine if a specific plasmid clone matches its reference sequence rightly to be accepted for use in experiments involving protein expressions.

6. qPCR assessment of 5hydroxymethylcytosine

The qPCR result of various IDH2 gene is shown, this can be glycosylated or non- glycosylated. With or without Mspl enzyme treatment.

The method was primarily used to assess the percentage of 5-hmc in IDH2. They were either glycosylated or non-glycosylated, with or without the activity of the enzyme. The double stranded DNA dissociation characteristics during heating was assessed using the melting curve.

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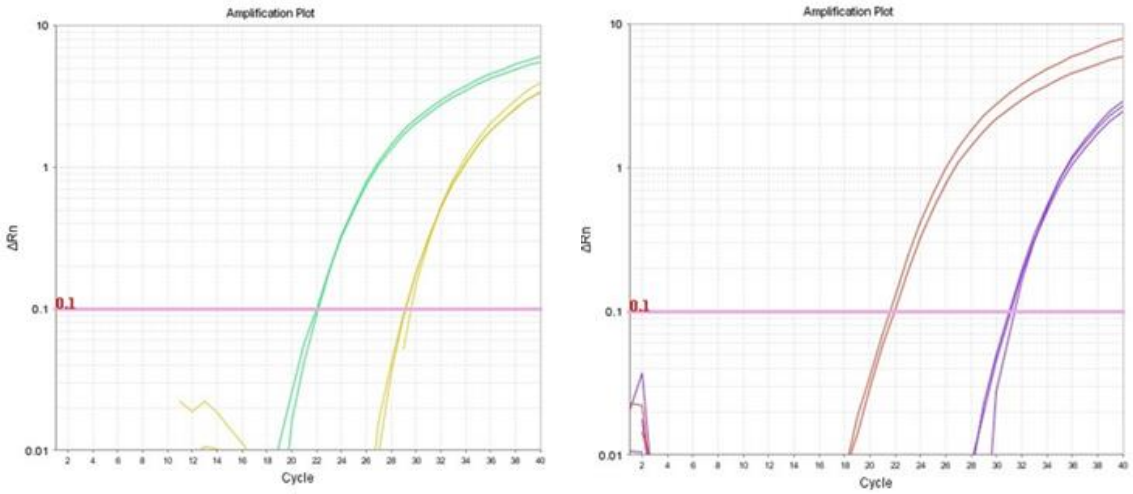
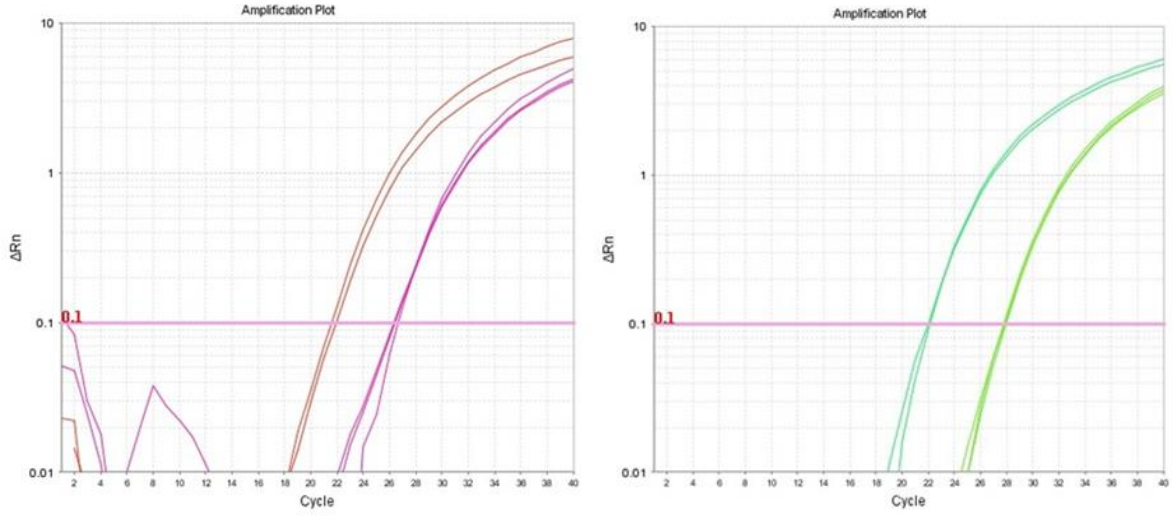


Figure (15): illustrates the comparison of the 5-hmc between IDH2 in glycosylated and non-glycosylated controlled conditions. A threshold (intersection) is represented and the difference in number of cycles corresponds to the 100% of the cell activity. The difference represents the amount of DNA action which is blocked by 100% of HMC.



Figure(16): Illustrates the comparison of 5-hmc between INS-1 dCAS9-Tet + guide IDH2 in glycosylated and non-glycosylated protected conditions. This compares the status 5-HMC between INS-1 status which is protected. The graph shows that the difference in the amount of DNA protected action is 0%.

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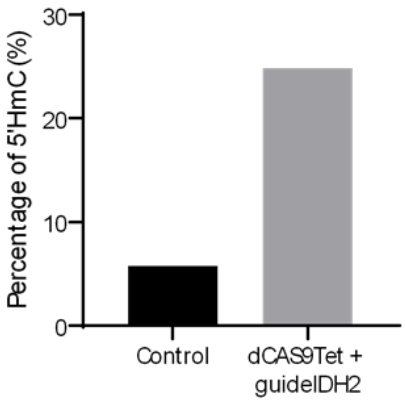
7. Percentage in controlled condition



Figure(17): This shows the comparison between the status hydroxymethylcytosine, the control and dCAS9-TET + guide IDH-2. The status of HMC was assessed in percentage on the cells treated with dCAS9-TET IDA-2 at 24% and 3.15% in controlled condition.

8. Melting curve

A melting curve analysis was done to check the absence of contamination and amplicon, see Figure 19. Values are between 88c and 92c. Melting temperature is the temperature at which about 50% of DNA is denatured. As temperature is increased the double strand dissociates causing an increased absorbance intensity, hyperchromicity.

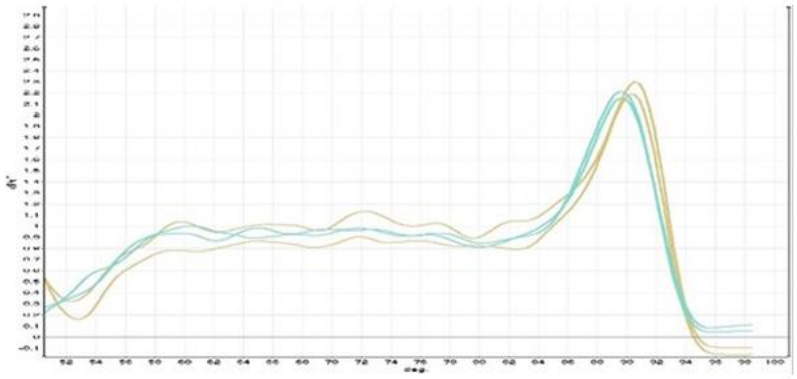


Figure (18): Shows a melting curve, the percentage of 5-hmc in controlled and dCAS9Tet + guide IDH2 conditions

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9. Western blot

Western blot involves the transfer of molecules which depends on protein diffusion out of the gel matrix which is followed by subsequent absorption to the transfer membrane in blotting. Absorbed protein removal from solutions helps in the sustenance of the concentration gradient that pushes protein to the membrane. Figure 30: Shows the transfer of protein membrane after transfer. It highlights dCAS9-TET ctrl, dCAS9-TET gIDH2 and GAPDH. Transfection using IDH2 showed bands of the same membrane

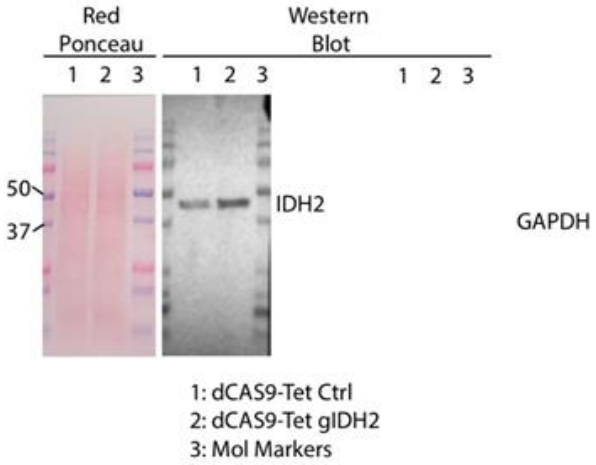

Figure (19): Shows Western blot transfer.

DISCUSSION

The different dCAS9-TET demethylase assays were considered for the purpose of investigating epigenetic modifications involving diabetes. TET protein fusion genetic tool was initially created and later validated, this was used in the modification of the epigenetics of the IDH-2 gene and CPG islands. The TET protein fusion genetic tool is a tool used by most of the CRISPR CAS9 systems, following the demethylation process. Increase in TET enzymes resulted in the successful purification of good quality recombinant proteins. Transfection with dCAS9-TET demethylase plasmid system on mammalian cells also occurred. DNA isolation and the evaluation of 5-hydroxymethylcytosine also followed. This was carried out using qPCR amplification; verification of expressed genes was done by western blot.

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The CRISPR-CAS9 tool was adopted in multiple genomic DNA sequence, these were simultaneously targeted and appeared easy. These allowed the specific demethylation of gene promoters, leading to epigenetic slicing. This was also applied to different regulatory elements. The CRISPR-CAS9 tool was used on all 20 bp genomic DNA sequences, further followed by the PAM sequence. Target genes were also identified using the SnapGene software as shown in Figure 9. This also showed CpG in green, a colour showing epigenetic alteration that affects a different gene while allowing the remaining DNA unchanged, this was denoted by CCGG. The grey box showed CCGG sequences, as shown in Figure 10.

The data gathered from this research work may help in understanding the epigenetic mechanism of diseases that are attributable to the mutation of the IDH-2 gene and diabetes. Effective means were used to obtain the plasmids bearing the special single RNA from the DNA primers. The outcome of the sequencing apparently indicated that the plasmid inlets were pLentiPuro Guide-IDH-2, eGFP and dCAS9-TET as highlighted in Figures 10 and 11. The result acquired form the Sanger sequencing clearly showed that the specific inlet was used. To allow the increase of the CpG methylation of DNA sequence in the genome of mammalian cells, the CRISPR-Cas9 based tool was also used for the increase in the CpG methylation of the DNA sequence in the genome of mammalian cells. DNA methylation mostly happens at the cytosine of the CpG dinucleotides which tend to cluster in regions knowns as CpG islands. DNA methylation functions mostly by actively slicing genes and DNA regions where transcription isn't desired.

A miniprep culture was carried out to isolate plasmid DNA from bacteria. This is a quick process mostly used in molecular cloning for the analysis of the bacterial clones. A conventional plasmid DNA miniprep typically produces between 50 µg and 100 µg, this depends majorly on the strain of cells used, this method is convenient as the use of filter paper lysing subjects many plasmids to miniprep. Furthermore, by eluting the filter paper, the plasmids are collected and directly sequenced.

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Colony PCR which involved using double and triple sets of primer was used to facilitate agarose gel analysis of recombinants. The product generated determined the presence of cloned E. coli. The band used in the discernment of digestion was severed by Dr Sergio, ligation was further applied.

For the identification of the optimal INS-1 cell ratio during the process of cell transfection, pLPGRNA IDH2, eGFP and dCAS9-TET cherry plasmids were used. It was shown from the fluorescence microscopic images of the transfected cells that the INS-1PEI/DNA corresponded to the ideal transfection efficiency. Puromycin; an amino nucleoside antibiotic synthesized by Streptomyces alboniger was used to determine the type of INS-1 cell and this meant a titration curve was created. After incubation for a day, 0.5 µg /ml puromycin induced 80% apoptosis for the INS-1 cells. This meant that 0.5 µg /ml was the ideal concentration needed for the selection of transfected INS-1 cells with dCAS9-DNMT3A (methylase), dCAS9- TET (demethylase) and the gRNA.

Result qPCR

The threshold of the real-time PCR reaction is defined as the level of signal by which a statistically significant increase is reflected over the baseline signal which is calculated. This is used to distinguish amplification signals that are relevant from the background signal. The real-time PCR instrument software automatically sets its threshold at 10 times the standard deviation of the fluorescence value of the baseline. The positioning of the threshold however can be adjusted at any point in the exponential phase of PCR.

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Threshold cycle (Ct)

This is the threshold cycle and it is the cycle number at which fluorescent signals of the reaction is able to cross a threshold. The threshold cycle is used in the calculation of the initial DNA copy number, this value is indirectly related to the starting amount of target. For example, when real time PCR results are compared from samples containing different amounts of a target, the sample which possesses twice the starting amount will produce a Ct which is one cycle earlier than a sample which contained half as many copies of the target before amplification.

This might mean that the PCR is 100% efficient (the amount of product perfectly doubles during each cycle) in both reactions.

Western blot; a method that allows for the separation and the identification of proteins on the basis of molecular weight using gel electrophoresis (Yang and Mahmood, 2012) was used in this research project. The western blot showed a band for each type of protein obtained through a separation membrane. After the process of transfection, a significant amount of protein was discovered according to the western blot outcomes for protein concentration. The main aim for carrying out a western blot analysis was to identify the protein following the use of IDH-2, and other antibodies namely anti-rabbit and anti-mouse.

CONCLUSIN

this research provided an excellent overview of the current status of epigenetic studies in diabetes research. Diabetes is caused by multiple factors, involving interactions between genetic and environmental factors. Research has shown a rapid increase between the rate of diabetes and associated complications; therefore, additional methods are needed to reduce this trend. The scope of epigenetics and epigenomics associated with diabetes though still not well understood is developing spontaneously and many groups are working on the generation of a well and clinically defined cell type-specific epigenome atlas.

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However, epigenetics plays a major role in the growing incidence of type 2 diabetes, this will be a great challenge to dissect over the next few years, the role of histone modifications and DNA methylation in the pathogenesis of the disease and its complications will be a great challenge to dissect.

Two additional vital questions requiring answers are whether the next generations can inherit the epigenetic changes induced by today's sedentary lifestyle and if these changes are reversible. Several epigenetic drugs are currently being tested in clinical trials, most in use already; it may, therefore, be possible to test epigenetic drugs as putative novel drugs for the treatment of diabetes and its complications. The field of Epigenetics remains in its early days, it is expected that further studies will define the interaction of genetic and non-genetic effects which leads to tissue-specific destruction, thereby enhancing the ability to predict and possibly change epigenetic processes.

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