RESEARCH ARTICLE

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Genetically engineered insulin mediated by glucose transporter-2 (GLUT2) promoter for the biosynthesis of insulin in rat hepatocytes: Insulin gene therapy

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ABSTRACT

The expression of insulin in hepatocytes by hepatic gene therapy is a promising treatment for diabetes. The conversion of immature proinsulin to mature insulin occurs only in cells that contain the enzymes responsible for the cleavage of proinsulin to insulin. For successful hepatic insulin gene therapy, insulin production must be tightly coupled to glucose concentration. Hepatocytes are excellent target cells for insulin gene therapy since, they are similar to pancreatic beta cells, they have the ability to rapidly adapt to blood glucose concentrations as they possess glucose-sensing components, such as Glucose Transporter-2 (GLUT2). So, we engineered rat proinsulin with the sites of cleavage (Furin Cleavable Sites) using site-directed mutagenesis for the removal of C-peptide to form the two chains A and B for mature insulin production. This engineered proinsulin was constructed into a non-viral expressing vector and regulated by glucose transporter-2 promoter (GLUT2 promoter) to control the amount of mature insulin expressed and to modulate the amount of glucose found in hepatocytes. The mature, active, and regulated expressed insulin was secreted according to the amount of glucose-regulated by the glucose transporter-2 (GLUT2) promoter.

Key words: Gene therapy, GLUT2 promoter, hepatocytes, insulin, mutation, preproinsulin, transfection, vector.

Introduction

Diabetes mellitus (DM) is a chronic disease that occurs when the body cannot produce enough insulin or cannot use insulin effectively (Harris & Zimmet, 1985). It is characterized by chronic hyperglycemia together with disturbances of carbohydrate, fat, and protein metabolism resulting from defects of insulin secretion, insulin action, or both (WHO, 1999). Insulin is a hormone produced in the pancreas that stimulates glucose uptake from the food from blood to enter the body's cells where it is converted into energy needed by muscles and tissues to function (Meslier et al., 2003). Insulin is a 5808-Da two-chain protein that is produced from a single-chain precursor, proinsulin, in the pancreatic beta cells of all mammals (Barcinskiand & Rosenthal, 1977). The proteolytic enzymes immediately cleave the signal peptide, generating proinsulin (Ashcroft et al., 1978). proinsulin, comprising three peptides linked by two pairs of basic residues in the following order: Bchain-Arg-Arg-Cpeptide-Lys-Arg Achain (Schwartz, 1990). C peptide is cleaved off from proinsulin at the adjacent dibasic residues during its transport through the *trans-Golgi* networks to immature-type secretory vesicles (Orci et al., 1987).

Gene therapy as a novel field of medicine holds tremendous therapeutic potential for a variety of human diseases including Insulin-dependent Diabetes Mellitus (IDDM) (Selden et al., 1987; Docherty, 1997; Hohmeier et al., 1997; Nett et al., 2003; Tuch et al., 2003). The beta (β) cell has the ability to regulate insulin production at the transcriptional, post-transcriptional, translational, and post-translational levels, as well as the ability to store and secrete insulin in a highly regulated fashion. In response to glucose, β cells can sense and quickly respond to small changes in circulating glucose levels over a broad range of physiological concentrations (2–20 mM) through concentration-dependent entry and metabolism of glucose (Won et al., 2009); they do so through the activity of glucose transporter-2 (GLUT2) and glucokinase. GLUT2 is a trans-membrane protein that enables glucose transport across cell membranes, whereas glucokinase is an enzyme that phosphorylates glucose to initiate its intracellular metabolism. GLUT2 and glucokinase have been dubbed the "glucose sensors" of β cells because they enable β cells to sense glucose over a very broad range of concentrations. They can do so due to their high Km for glucose (~17 and 8 mM, respectively), which allows their activity to vary substantially based on glucose availability (Newgard & McGarry, 1995 Won et al., 2009).

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For insulin gene therapy to be achieved, several criteria must be considered. First, the appropriate cells must be targeted for insulin production. At a minimum, these cells would need to express the glucose sensors, GLUT2, and glucokinase. Second, insulin transgene expression must be responsive to fluctuating blood glucose levels, being upregulated during hyperglycemia and downregulated during euglycemia. Third, there must be some mechanism in place for the target cell to process proinsulin into mature insulin. Lastly, an appropriate gene correction tool must be utilized to safely and effectively drive long-term insulin expression (Handrof, Sollinger & Allam, 2016). The more active site in the GLUT2 promoter which is more active in the primary rat hepatocytes is from -500 to +240 (Mitchell & Tjian, 1989; Neuberg et al., 1989; Ahn et al., 1995). For engineering insulin-producing capacity, two types of cells are available: neuroendocrine and non-neuroendocrine. Upon extracellular stimulation, neuroendocrine cells exocytose secretory granules containing a mature bioactive peptide hormone that is converted from its precursor propeptide. This cell type may correspond to the mode of action of rapid-acting insulin (Lu et al., 1998). On the other hand, non-neuroendocrine cells, such as hepatocytes or muscle cells, are easily obtained, but unlike neuroendocrine cells, must be engineered with a processing mechanism and a regulatory secretion system (Kahn & Weir, 1994). The most commonly chosen target cells of insulin gene therapy are hepatocytes, which are attractive targets for insulin expression because they are closely related to β cells developmentally, play a very important role in glucose homeostasis, and are relatively easy to target (The Diabetes Control and complications, 1993; Hovorka, 2011; Vehik, 2013).

A weaker tissue-specific promoter has been employed for hepatic insulin gene therapy to not only reduce the potential for hypoglycemia but also to improve targeting to the tissue of choice. The liver-specific GLUT2 promoter drives insulin gene expression in a glucose-inducible but insulin-repressive fashion and found an improvement in diabetic hyperglycemia (Burkhardt et al., 2005). The non-viral method was used to drive the expression of insulin into hepatocytes because it is safer and includes less of an immune response.

Adult rat hepatocytes in short-term primary culture can respond to hormones and nutrients as well as they do in vivo and often present a higher level of transcription of liver-specific genes than differentiated hepatoma cells (Jefferson et al., 1984).

Materials and Methods

Construction of the mutated rat preproinsulin cDNA

The genomic rat DNA was extracted from the liver by TIANamp genomic DNA extraction kit (TIANgen kit), amplification of the gene of interest by PCR using 2xTagPCR Mastermix using the designed preproinsulin primers;

Forward:
CATGGCCCTGTGGATGCGCTTCCTGCCCCTG

Reverse:
GAGTTGCAGTAGTTCTCCAGTTGGTAGAGGA

The amplified gene was run on agarose gel electrophoresis to confirm the band size of the amplified preproinsulin gene (about 333bp), then sequenced and introduced to the linearized cloning vector (NEB PCR Cloning Kit#E1202). After cloning the preproinsulin inside was ready for the mutation (Q5Site-DirectedMutagenesis Kit# E0554S) to create the two furin cleavable sites inside the preproinsulin gene to get the mutated preproinsulin.

A variety of site-directed mutations to engineer proinsulin to be a substrate for furin enzyme. This enzyme (also known as Paired basic Amino acid Cleaving Enzyme, PACE) is a Golgi-associated propeptide endoprotease that is present in the constitutive secretory pathway of virtually all cells. The introduction of furin consensus sequences at the B-chain/C-peptide and C-peptide/A-chain junctions to create tetra basic furin cleavage site. So**,** we engineered a cDNA for furin-cleavable rat proinsulin that will be efficiently processed to mature insulin. To perform this mutation in the preproinsulin which is cloned inside the cloning vector, we needed to make a mutation by substitution using a Q5 Site-Directed Mutagenesis Kit (#E0554S) to change the three mentioned amino acids (glutamic acid to lysine, valine to arginine in the C-peptide connecting to B-chain, and glutamine to arginine in the C-peptide connecting to A-chain) (Figures 1 and 2).

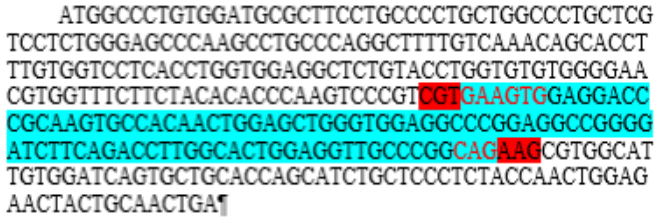

Figure 1. *Wild type insulin gene sequences.*

Figure 1. *Mutant insulin gene sequences.*

The primers for mutation were designed as follows according to the NEBaseChanger, which could be found at NEBaseChanger.neb.com.

The furin-cleavable consensus sequence at the B-chain/C-peptide;

Forward primer:
5'CAAGTCCCGTCGTAAACGGGAGGACCCGCAAG-3'
Reverse Primer:
5'GGTGTGTAGAAGAAACCACGTTCCCC-3'
The furin cleavable consensus sequence at the C-peptide/A-chain junction;
Forward primer:
5'GAGGTTGCCCGGCGGAAGCGTGGCATTG 3'.
Reverse primer:
5'CAGTGCCAAGGTCTGAAGATCCC 3'
After mutation, we had a vector containing the mutated preproinsulin which was ready for transformation to get high yield. For confirmation of the correct insert of the mutated preproinsulin, we amplified the insert using forward and reverse primers containing the site for ligation in the plasmid, purified and desalted.
Forward primer:
GATCCaccATGGCCCTGTGGATGCGCTTCCTGCCCCTG
Reverse primer:
AATTCGTTGCAGTAGTTCTCCAGTTGGTAGAGGGA

Amplification of murine glucose transporter-2 promoter

The GLUT2 gene is 33,980 bp and its promoter is too long. The more active region in the rat GLUT2 promoter for rat hepatocyte culture is from -500 to +240 (about 740 bp) (Figure 3). So, this region was amplified by PCR from the extracted rat genomic DNA. PCR primers are designed to incorporate appropriate restriction sites at the ends of the target DNA for subsequent digestion and ligation into the vector of choice. The addition of restriction sites by PCR during amplification of a genetic sequence allows for the insertion of virtually any target gene into any vector.
Forward primer:
5'CGATAACAATCTTGATTTCCACATCACAAAC-3'
Reverse primer:
5'AGCTTTGCAGGCTGAGGCTGGAGGGAGGCTGGA-3'.

Construction of the expressing plasmid

The expressing plasmid (#13031) (pcDNA3-EGFP) was received from the Addgene, and was ready to be isolated, purified, modified, and digested (Figure 4). This plasmid is 6160 bp and has an EGFP (enhanced green fluorescent protein) that was ready to be fused to the preproinsulin insert to detect the inserted gene location by its green fluorescent appearance. The plasmid DNA was isolated from bacteria, and purified to be digested for the insertion process. The digestion happened to the purified plasmid by ECORI and BamHI restriction enzymes, then purified and desalted by using a purification kit (Qiaex II Extraction Kit #20021), then the ligation step for the purified plasmid and the designed mutated preproinsulin gene proceeded. The resulting ligated plasmid was also digested by NruI and HindIII restriction enzymes, for the GLUT2 promoter ligation. So, we had a new construct of pcDNA3-GLUT2-mutated preproinsulin-EGFP ready to measure by UV spectrophotometer and transfected into cells.

-500 to +240 promoter region
TAACAATCTTGATTTCCACATCACAAACGTGCAATTGGACTAGAGG
ACCAGAGAGGCCAGGCAGGGGGACCTGACCTTCAGGACGGAGTTC
TTCCCGTGAAGAACTTGGTTTTCACTCCTGCCTCTACTCTTATCTGA
CTCAACAGGAGAAGAACGCAGCAGGCTTCTCTGAGCTGTTTCCTCT
GATTTATAGACAAAACAAAATTTAAAAACCCATTCCTGTAGCTTTG
CTAAAAAAAAGACAATGAAGGGGAGAAAGGGAGGCGAGGTTACC
ATTTCCGATTCTAGGTGTTTCCTCTCTTAATTAACGATGTAATCAGG
ATGTGACCTGAAAGGTTTTAAAGTATAAGGCCAGGTCTATGGAGG
ACACCTGCTGGGTCAGAACATTTGCTCTGCAAACACAAGGACCTG
AACTCAATCGCTAGTACCCAGTACGGGTTGTGCCTGCATAGAACGG
GCACAGGGGCACCGAGGCACCGAGACAGAGGGATCATAATCAGA
GCTGGTTTTGCAGTGCAGCAGGAATCTCTGAGTGCTTTTGAGAACT
CAGTAGGTAGACGTCATCCCAGGGCAAAGTACAAAGAGCCAGGTT
TCCTTGCCAGACATTATCCCACAGAAACCCGCAGCTCCTCTGTGCG
CCCTGAAGCCACTGTAGCAGTTTCGCATGAGGTTGGTCTGTGACCT
GAATTCACCTGGTAAAATCAGCCTGTGCAATATCCTCCCTCCAGCC
TCAGCCTGCA

Figure 3. *The sequence for the more active region in rat GLUT2 promoter for rat hepatocyte culture.*

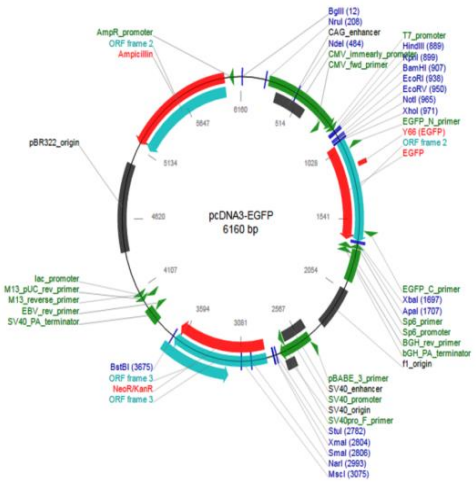

Figure 4. *Construction of plasmid #13031 received from the addgene.*

Primary culture of rat hepatic cells

According to Li et al., (2010), with some modifications, rat hepatic cells were purchased from Cell Biologics, and shipped in suspension (catalog no. RA-6224F). Cells expanded on multi-well culture plates ready for experiments under the cell culture conditions specified by Cell Biologics.

The plateable cells were derived from pooled or single donors. Viabilities generally > 75%. Centrifuged and suspended the cells in 30 ml warm William's complete Medium (added the following to Williams' Medium E: L-

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glutamine to 2 mM, fetal calf serum (FCS) to 5%, dexamethasone to 100 nM, cadmeium chloride to 9 µg/l, DMSO to 0.5%, penicillin to 100 IU/ml and streptomycin to 100 mg/ml), and then centrifuged and re-suspended again in 20 ml warm William's complete Medium and counted the cells within the cell suspension using a hemocytometer and determined cell viability by trypan blue staining.

Plate cells at a desired volume on cell culture collagen-coated plates. Cultured the cells at 37°C in a humidified atmosphere of 95% air and 5% CO_2. After 4h culture, the cells could either remain in the same serum-containing medium or replace the medium with serum-free medium, HepatoZYME-SFM. The serum-free medium helped to maintain cell morphology with no adverse effects from hormones when a serum-free medium is used. Allow cells to recover and grow at least overnight before experimentation. They replaced the growth medium at 2-day intervals, if needed (Li et al., 2010). After the hepatocyte culture condition was optimized, the cells every day under an inverted microscope to detect the hepatocyte viability, division, and morphology. Imaged every 2 h on the first day of culture and every day using (Olympus inverted microscope). After optimization of the cell culture condition, transfection takes place using 2 µl turbojet transfection reagent (# R0531) in 1 ml culture medium after 24 h of culture.

Examination after transfection under an inverted green fluorescent microscope

After the transfection, the cells were examined under an Olympus inverted microscope, the transfected cells appeared in green fluorescent color.

Examination of the expressed fused protein

The transfected hepatocytes were exposed to different concentrations of glucose (from free to 25 mM glucose), analyze the amount of glucose transported into and out of the cells in cell lysates and media by glucose colorimetric assay method (Glucose (GO) Assay Kit #GAGO-20). For the measurements of the amount of insulin released against the different concentrations of glucose added, we used also the same cell lysate and media from each separate concentration of glucose to measure by cohesion Rat Insulin ELISA Kit #CEK1622.

Sequencing

The PCR products and plasmid construct were sequenced by the use of ABI prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Germany).

Results

To get the rat preproinsulin, we extracted the genomic rat DNA from the spleen, liver and pancreas. Then, we ran about 10 µl from the extracted genomic DNA from different tissues to obtain the perfect one, as shown in Figure 5.

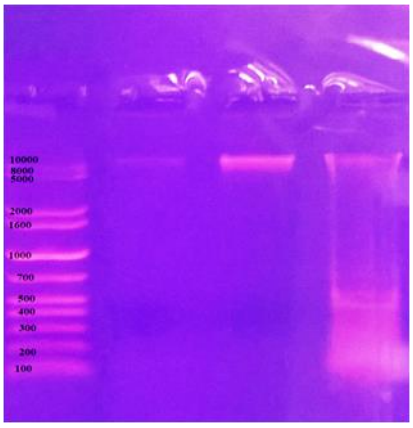

Figure 5. *The genomic DNA extracted from rat. Lane 1: The DNA ladder, lane 2: The DNA extracted from rat spleen, lane 3: The DNA extracted from rat liver, and lane 4: the DNA extracted from rat pancreas.*

The genomic DNA extracted from rat liver showed a good band after running on 1% agarose. Then, we amplified the rat preproinsulin gene from the rat liver genomic DNA by PCR amplification technique using the specific primer designed for the rat preproinsulin gene. The amplified PCR product was run on 1% agarose to indicate the gene size band (about 333 bp). The amplified product was also sequenced to confirm the sequence for the preproinsulin gene (Figure 6).

Figure 6. *The forward sequence of the amplified PCR product (the rat preproinsulin gene).*

The amplified preproinsulin gene was inserted into the linearized cloning vector NEB#1202 to be ready for the mutation step. Mutation proceeded by using the designed primers for mutation in the two chains. After mutation, we plated the resulting vector on X-gal and IPTG selection plates to determine which colonies contained the mutation by the white and blue colonies. Then amplified the mutated preproinsulin, and inserted it into the vector, we ran sequencing to be sure that the preproinsulin had mutated perfectly. Next, from the genomic DNA, we also amplified the more active region in the glucose transporter-2(GLUT2) promoter for the hepatocyte culture and inserted it into the vector. Thus, we obtained a vector containing the mutated preproinsulin gene derived by the GLUT2 promoter which we

inserted (PCDNA3-GLUT2-mutated preproinsulin-EGFP). This vector concentration was measured to detect its purity (Table 1), and sequenced for both forward and reverse sequences to be sure of the location of the promoter and the inserted gene.

Table 1. *Measuring the concentration and the purity of the extracted plasmid using UV spectrophotometer (the dilution factor was X300).*

Plasmid	A260	A280	Concentration (µg/ml)	Ratio
PCDNA3-GLUT2-mutated preproinsulin - EGFP	0.258	0.143	3870	1.8

The plasmid was ready to transfect into the primary rat hepatic cells. Primary rat hepatocytes washed, diluted, counted (Figure 7 and Table 2), then cultured in 12 well coated collagen plates with complete Williams E medium in a humified atmosphere incubator for 4 h, and after that, the medium was replaced by hepatozyme, a serum free medium to demonstrate the morphology of the cells (Figure 8).

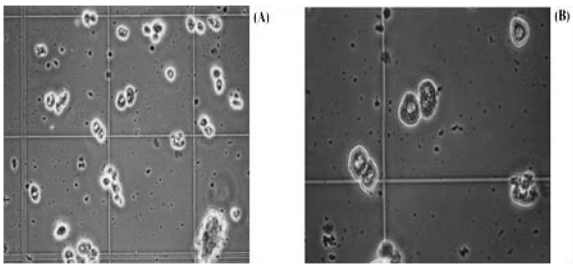

Figure 7. *The count of the rat hepatocytes on haemocytometer using trypan blue under inverted microscope. (A) The rat hepatocytes X100. (B)The rat hepatocytesX200.*

Table 2. *The count of live and dead cells on haemocytometer to detect the viability and cell density of the rat hepatic cells. Viable cells = 71.5 X 10^4 X 2 = 143 X 10^4 cells/ml. Total count = 75.5 X10^4 X 2 = 151 X10 ^{4} cells/ml.*
Viability % = viable/total =143/151 =95 %
Cell density = total count

# of square	Live cells	Dead cells	Total count
1	75	0	75
2	62	7	69
3	91	3	94
4	58	6	64
Average	71.5	4	75.5

The rat hepatic cells were investigated after plating under the inverted microscope every 24 h to detect the cell viability, division and morphology (Figure 9, Figure 10 and Figure 11). After optimization the cell culture conditions, transfection took place using 2 µl turbojet transfection reagent in 1 ml culture medium, then added 0.5 µl from the prepared expressing plasmid and let for 7 h inside the incubator.

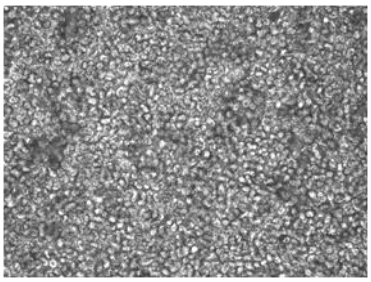

Figure 8. *The rat hepatocytes immediately after plating on a 12-well collagen coated plate with Williams E complete medium under inverted microscope, shows the cells X100.*

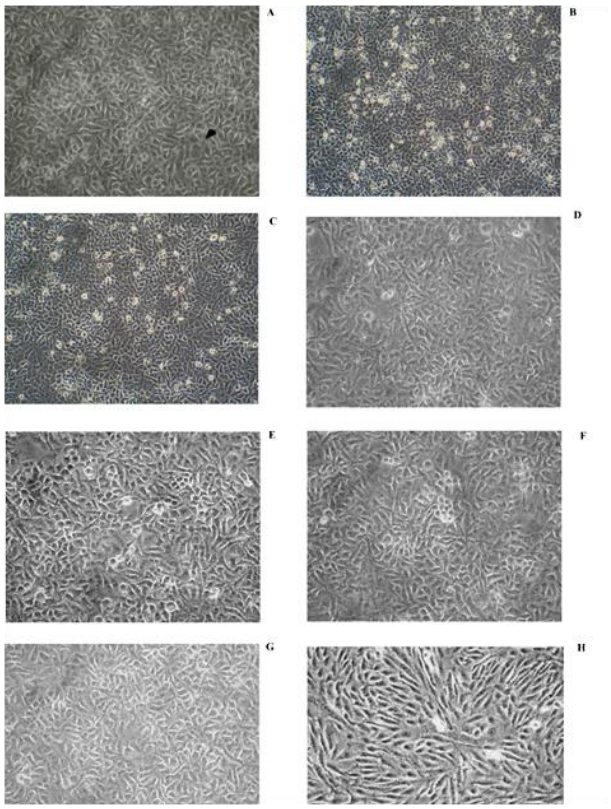

Figure 9. *X100 rat hepatic cells after plating. (A) After 24 h of plating. (B) After 48 h of plating. (C) After 3 days of plating. (D) After 4 days of plating. (E) After 5 days of plating. (F) After 6 days of plating. (G) After 7 days of plating. (H) After 9 days of plating.*

Next, started to examine the cell morphology and viability after transfection, analyze the expression of the fluorescent fused protein by visualization under inverted green fluorescent microscope. Although the GLUT2 promoter is a weaker promoter than others, the rat hepatocytes transfected with a construct controlled by this promoter could not live longer than the healthy cells (Figure 12 and Figure 13).

We examined the released fused protein under the green fluorescent protein using PCDNA3-GLUT2-mutated

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proinsulin-EGFP in both media and inside the cell under the inverted green fluorescent microscope, and we examined the transfected rat hepatocytes with PCDNA3-GLUT2-mutated preproinsulin-EGFP and its media in case of stimulation with free glucose and different amount of glucose (5 mM and 25 mM). In stimulation with glucose, the green fused protein crosses the cell membrane out to the media relating to the amount of glucose found (Figure 14).

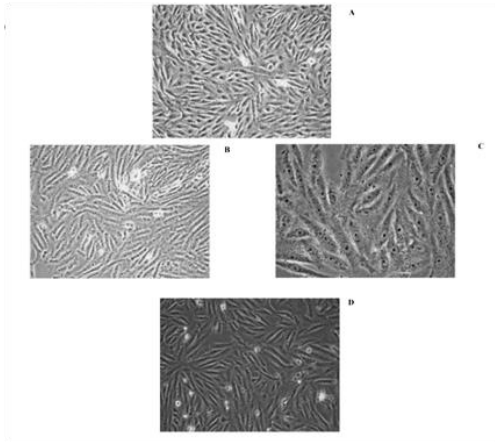

Figure 10. *(A)X100 rat hepatic cells after 10 days of plating. (B)X100 rat hepatic cells after 11 days of plating. (C)X200 rat hepatic cells after 11 days of plating. (D)X100 rat hepatic cells after 13 days of plating.*

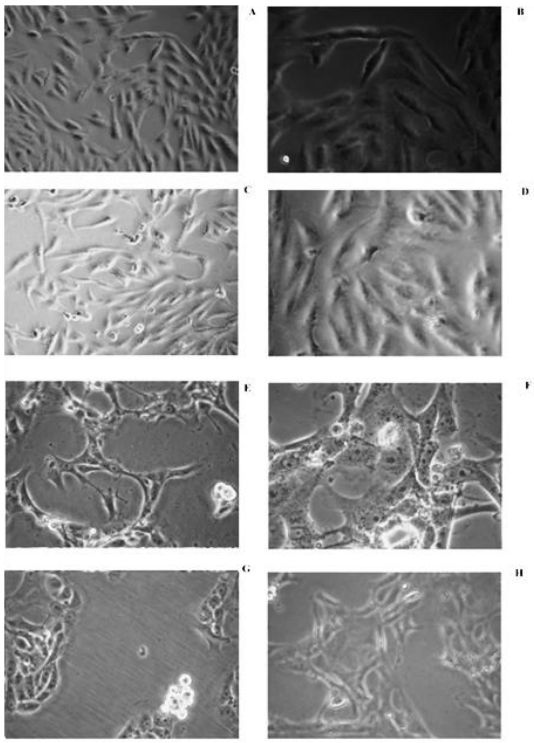

Figure 11. *(A)X200 rat hepatic cell after 16 days of plating. (B)X400 rat hepatic cell after 16 days of plating. (C)X200 rat hepatic cell after 17 days of plating. (D)X400 rat hepatic cell after 17 days of plating. (E)X200 rat hepatic cells after 19 days of plating. (F)X400 rat hepatic cells after 19 days of plating. (G)X200 rat hepatic cells after 20 days of plating. (H)X400 rat hepatic cells after 20 days of plating.*

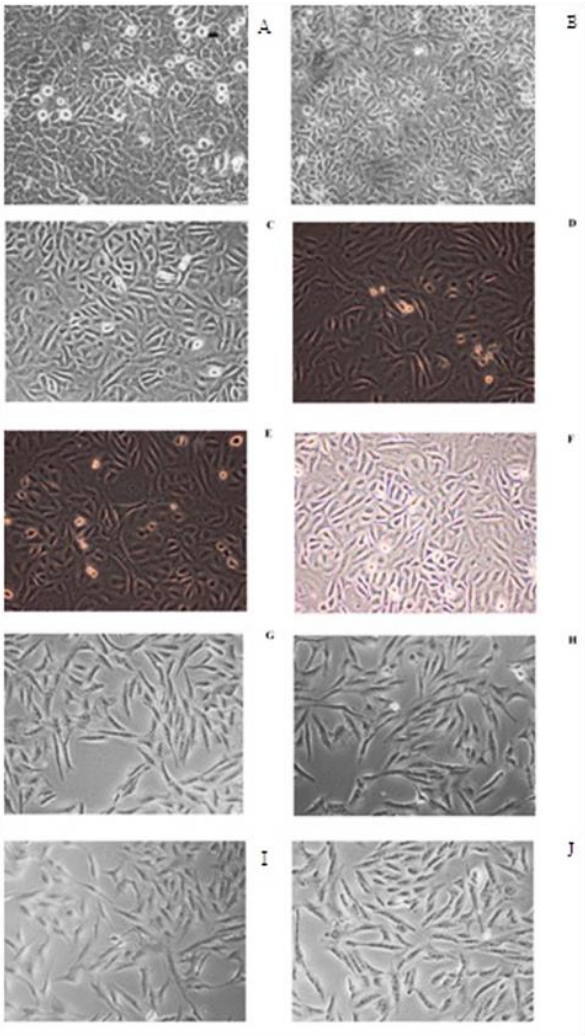

Figure 12. *The rat hepatic cells after the transfection with a construct controlled by GLUT2 promoter (PCDNA3-GLUT2-mutated preproinsulin- EGFP) X100. (A) The rat hepatocytes after 7 h from transfection. (B) The rat hepatocytes after 24 h from transfection. (C) The rat hepatocytes after 2 days from transfection. (D) The rat hepatocytes after 3 days from transfection. (E) The rat hepatocytes after 4 days from transfection. (F) The rat hepatocytes after 5 days from transfection. (G) The rat hepatocytes after 6 days from transfection. (H) The rat hepatocytes after 7 days from transfection. (I) The rat hepatocytes after 8 days from transfection. (J) The rat hepatocytes after 9 days from transfection.*

In the case of the addition of 5 mM, and 25 mM glucose to the media, the cells increased the transport of glucose gradually, as the strongest action of the GLUT2 promoter was in the case of using 25 mM glucose (Figure 15 and Figure 16).

Glucose was measured in cell lysates and cell media of transfected culture cells three times and obtained the average after different concentrations of glucose (0-25 mM) were added to the media by colorimetric glucose assay (Table 3).

Due to the amount of glucose regulated and released, the insulin is also released from the construct: PCDNA3-GLUT2-mutated preproinsulin-EGFP. So, we measured the amount of

insulin released from this construct three times and obtained the average to balance the amount of glucose found.

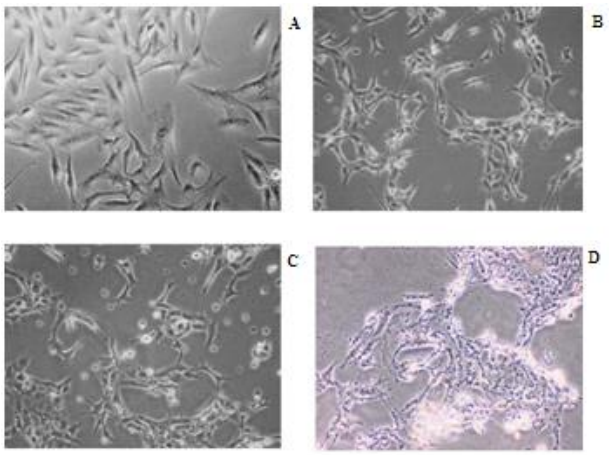

Figure 13. *The rat hepatic cells after transfection with a construct controlled by GLUT2 promoter (PCDNA3-GLUT2 -mutated preproinsulin-EGFP) X100. (A) The rat hepatocytes after 10 days from transfection. (B) The rat hepatocytes after 11 days from transfection. (C) The rat hepatocytes after 12 days from transfection. (D) The rat hepatocytes after 13 days from transfection.*

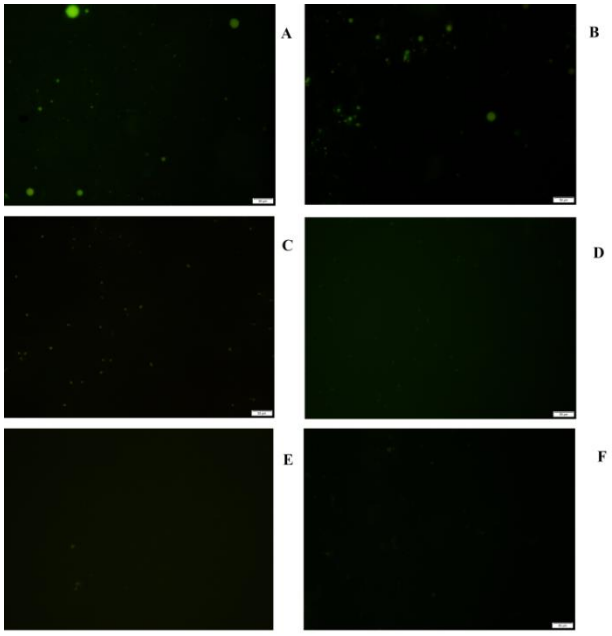

Figure 14. *The media of stimulated transfected rat hepatocytes with PCDNA3- GLUT2- mutated preproinsulin-EGFP X100. (A) The media after 24h from transfection. (B) The media after 3 days from transfection. (C) The media after 5 days from transfection. (D) The media after 7 days from transfection. (E) The media after 10 days from transfection. (F) The media after 13 days from transfection.*

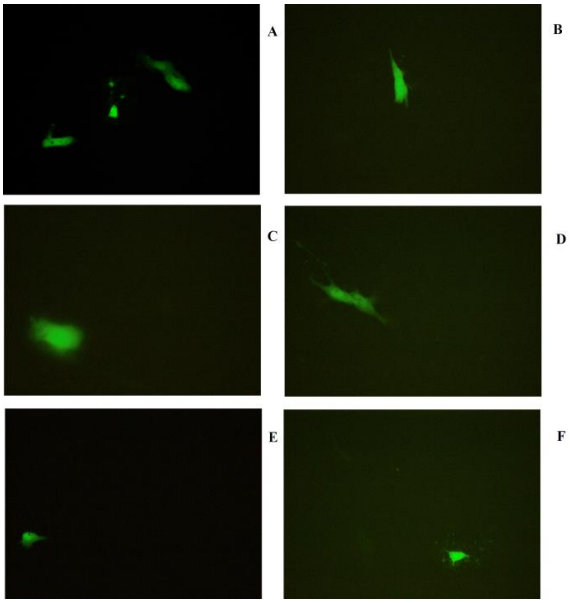

Figure 15. *The transfected rat hepatocytes with PCDNA3-GLUT2-mutated preproinsulin-EGFP when 5 mM glucose added to the media X200. (A) The transfected rat hepatocytes after 24 h from transfection. (B) The transfected rat hepatocytes after 3 days from transfection. (C) The transfected rat hepatocytes after 5 days from transfection. (D) The transfected rat hepatocytes 7 days from transfection. (E) The transfected rat hepatocytes after 10 days from transfection. (F) The transfected rat hepatocytes after 12 days from transfection.*

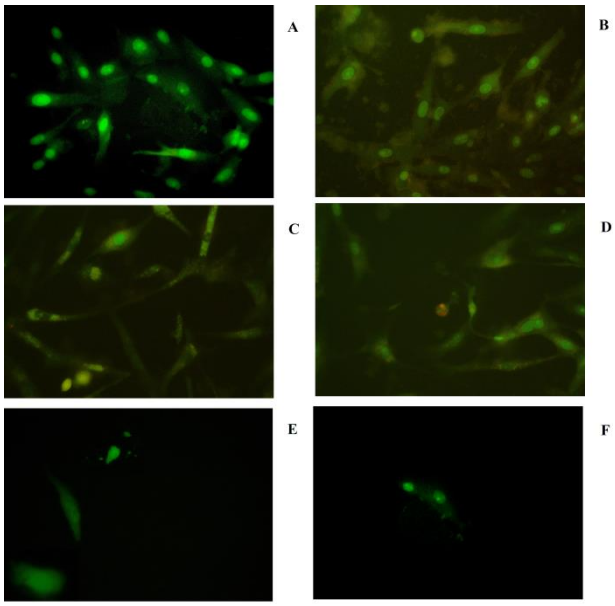

Figure 16. *The transfected rat hepatocytes with PCDNA3-GLUT2-mutated preproinsulin-EGFP when 25 mM glucose added to the media X200. (A) The transfected rat hepatocytes after 24h from transfection. (B) The transfected rat hepatocytes after 3 days from transfection. (C) The transfected rat hepatocytes after 5 days from transfection. (D) The transfected rat hepatocytes 7 days from transfection. (E) The transfected rat hepatocytes after 10 days from transfection. (F) The transfected rat hepatocytes after 12 days from transfection.*

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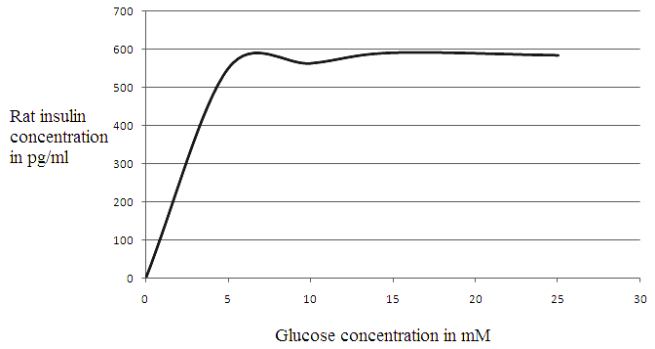
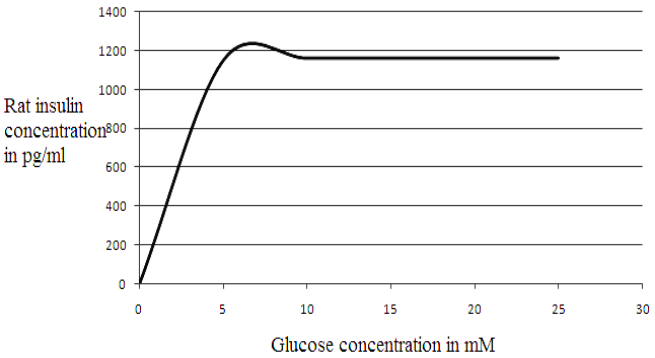
Table 3. The colorimetric glucose assay method for the detection of the amount of glucose present in both cell media and lysates after the addition of different amount concentrations of glucose to the media of transfected cells. This method shows how the cells can consume the amount of outer glucose in the media to cross the cell membrane. The GLUT2 promoter construct can transport the glucose in and out of the cells, as this promoter is a glucose transporter and regulator for the amount of glucose.

		Calorimetric glucose assay in mg/dl after 24 h from the addition of different doses of glucose in the transfected primary hepatocyte culture medium				
Different doses of glucose added in mM		**Free glucose**	**5 mM**	**10 mM**	**15 mM**	**25 mM**
PCDNA3-GLUT2-Mutated preproinsulin-EGFP	In media	ND	85	102	136	150
	In Cell lysate	ND	69	70	71	83



(in cell lysate)



(In media)

Figure 17. *The relation between the concentration of insulin released corresponding to different doses of glucose in the cell lysate and media using the PCDNA3-GLUT2-mutated preproinsulin-EGFP construct. The amount of insulin released increased as the amount of glucose increased in the case of cell lysate, in the case of the media The amount of insulin out to the media increased as the amount of glucose increased.*

Discussion

Proinsulin is transported in microvesicles to the Golgi apparatus, where it is packaged into membrane-bound vesicles known as secretory granules. Prohormone convertases 2 and 3 are responsible for the conversion from proinsulin to mature insulin, which removes the C peptide chain, liberating two cleavage dipeptides and finally yielding insulin. When we generated a furin cleavable site inside the preproinsulin gene inside the designing cassette to obtain insulin, it worked well as these cleavable sites do the action of PC1 and PC2 to remove the C-peptide and convert the immature insulin to a mature one, Proinsulin undergoes further maturation within secretory granules through the action of prohormone convertases PC1/3 and PC2, as well as carboxy peptidase-H. These enzymes are co-packaged with proinsulin in secretory granules and together act to remove C-peptide and produce mature insulin. However, prohormone convertases PC1/3 and PC2 are only found in β cells and other cells with the regulated secretory pathway, like pituitary cells intestinal K cells, and hepatocytes. Thus, for insulin gene therapy applications to be successful, it is important to maintain proinsulin processing, even if researchers choose to target cell types that do not have the regulated secretory pathway, like hepatocytes. In these instances, modifications can be made to the preproinsulin sequence to bypass the necessity of PC1/3 and PC2. The most commonly used modification is the incorporation of furin cleavage sites. Furin is a ubiquitously expressed endo protease that can efficiently cleave proteins at paired basic amino acid sites. Through the incorporation of furin cleavage sites, any cell of the body can produce fully functional insulin (Yanagita et al., 1993; Groskreutz et al., 1994).

GLUT2 is a transmembrane protein that enables glucose transport across cell membranes. GLUT2 and glucokinase have been dubbed the “glucose sensors” of β cells because they enable β cells to sense glucose over a very broad range of concentrations (Newgard & McGarry, 1995; Won et al., 2009). In our studies, GLUT2 showed its ability to sense glucose and transport it into and out of the cells. The present study was designed to investigate the relation between glucose and insulin and how they gather to make the balance inside and outside the cells, as there was a large amount of glucose outside the cells, the GLUT2 absorbs some of it inside the cells to make balance. Therefore, a certain amount of insulin is secreted according to the amount of glucose found. The obtained insulin secretion in our results derived from the GLUT2 promoter is much better than that derived from the strong promoter (Won et al., 2009; Andrew et al., 2016).

Insulin can be secreted from hepatocytes by performing some modifications to the preproinsulin gene to cleave correctly inside the hepatocytes and by activating a certain region in the glucose transporter-2 promoter in hepatocytes to sense and transport glucose. Hepatocytes are easily obtained

but must be engineered with a processing mechanism and a regulatory secretion system (Kahn & Weir, 1994). Rat hepatocyte is a good target to express insulin due to the presence of the GLUT2 gene which plays a perfect role in modulating glucose levels (Hovorka, 2011; Vehik et al., 2013). Although adult hepatocytes have a remarkable ability to proliferate in vivo, attempts to proliferate adult hepatocytes in vitro have been less successful (Overturf et al., 1997; Taub, 2014); these results are similar to our results as the count number of primary rat hepatocytes decreased every day till cell death. Thus, the primary hepatocytes could not divide well in vitro.

In my results, we supplemented the media of primary rat hepatocyte culture with insulin and dexamethasone to make the cells live longer. The supplementation of the media with hormones, such as dexamethasone and insulin, has greatly enhanced the attachment efficiency of hepatocytes and prolonged hepatocyte survival to a certain extent (Bissell et al, 1973; Michalopoulos & Pitot, 1975; Miyazaki et al., 1978)

A point of interest is that the use of a non-viral vehicle cassette is shown to be more efficient in in-vitro studies and safer when treating sensitive primary rat hepatocytes (Duckworth et al., 1998). SuperFect Transfection Reagent, TurboFect, Lipofectamine, Effectene Transfection Reagent, and Tfx-20 were the most effective for the transfection of primary hepatocytes and give comparable transfection efficiencies (Tang et al., 1996; Mosher & Crews, 1999). Similar results have been reported in our findings using the turbojet transfection reagent.

Conclusion

The current study aimed to produce insulin from other organs of the body by using a construction of the mutated rat proinsulin cDNA regulated by rat GLUT2 promoter transfected into hepatocyte cells cultured from rats to produce mature insulin as a strategy to modulate glucose levels after exposing the cells to different concentrations of glucose and measuring the amount of glucose consumed by the cells and the insulin amount produced.

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