INVESTIGATION OF THE MOLECULAR AND GENETIC RESPONSE IN ENTEROCYTES OF DUODENUM DURING ELEVATED INTRACELLULAR GLUCOSE LEVEL

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ABSTRACT

HOW DOES HIGH GLUCOSE AFFECT TO GENOME-WIDE mRNA EXPRESSION PROFILES IN FULLY DIFFERENTIATED HUMAN EPITHELIAL CELL CULTURE MODEL?

Glucose is one of the nutritional factor that involves in developing of obesity and type 2 diabetes in human. The studies indicated that enterocyte cells on intestine might play a role in dietary glucose sensing during obesity. Obese people are consumed high amount of dietary glucose and enterocyte cells consequently are exposed to high glucose. Thus, we aimed to find relevant physiological pathways and genome-wide mRNA expression profiles that can be regulated by glucose in fully differentiated human intestinal epithelial (CaCo-2). The cells were maintained two different glucose levels (5.5mM for control, 25mM for high glucose) at least three passages. The cells were grown on transwell system for 21 days to mimic human intestine system. Transepithelial electrical resistances (TEER) were measured to control monolayer formation and polarization. RNA isolation was performed and whole genome mRNA expression profile were determined following gene ontology analysis to find affected molecular pathways. Compared to control relative glucose level was found high in basolateral side of CaCo-2 cells that were under high glucose condition without effecting TEER. GLUT2, SGLT1, GLUT5 mRNA levels were significantly reduced during elevated glucose levels which is consistent with literature. Significant fold change analysis showed that 351 genes upregulated and 468 genes under high glucose condition. We found high glucose significantly leads changes of molecular pathways (downregulated; glycolysis and gluconeogenesis, adherens junction, fructose/mannose metabolism, pentose phosphate pathway and upregulated; protein export). These results provide us better understanding and open new window for glucose metabolism of enterocytes during obesity.

ÖZET

İNCE BAĞIRSAK ENTEROSİT HÜCRELERİNDE ARTAN HÜCRE İÇİ GLİKOZ MİKTARINA BAĞLI MOLEKÜLER VE GENETİK YANITIN ARAŞTIRILMASI

Glikoz, obezite ve tip 2 diyabet gelişimine neden olan beslenme faktörlerinden biridir. Araştırmalar gösteriyor ki ince bağırsaktaki enterosit hücreler obezitede glikozun hissedilmesinde rol oynayabilir. Obez insanlar yüksek oranda glikoz içeren besinler ile besleniyorlar ve bunun sonucunda enterosit hücreleri yüksek glikoza maruz kalıyor. Bu sebepten dolayı, farklılaşmış insan bağırsak hücresi CaCo-2 da glikoz ile bağlantılı fizyolojik yolakların ve tüm genom mRNA profilinin bulunması amaçlanmıştır. Hücreler iki farklı glikoz seviyesinde (5.5mM kontrol için, 25mM yüksek glikoz için) en az üç kere pasajlanmıştır. Hücreler transwell sistem üzerinde büyütülmüştür ve 21 gün sonra insan bağırsak sistemini taklit etmiştir. Hücrelerin oluşturdukları elektriksel direnç değeri, hücrelerin monolayer yapı ve polarize oluşumunu kontrol etmek için ölçülmüştür. Daha sonra RNA izolasyonu yapıldı ve tüm genom mRNA profili incelenip gen ontoloji analizine göre etkilenmiş metabolik yolaklar bulundu. GLUT2, SGLT1 ve GLUT5 mRNA seviyeleri yükselen glikoz seviyesi nedeniyle önemli derecede azaldı ki bu sonuçlar literatür ile de doğru orantılıdır. Yüksek glikoz ortamında 351 genin arttığı ve 468 genin de azaldığı görülmüştür. Yüksek glikozun önemli derece metabolik yolaklara etki ettiğini bulduk (azalanlar; glikoliz ve glikoneogenesis, fruktoz ve mannoz metabolizması, pentoz fosfat ve artan; protein export). Bu sonuçlar obezitede enterosit glikoz metabolizması için bize yeni pencereler açmayı sağlayacaktır.

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CHAPTER 1

INTRODUCTION

This part of first chapter will ensure a general overview of obesity and then followed by risk factors including genetic, hormonal, and nutritional that leads obesity. The role of enterocyte on nutrient sensing will be discussed in last part of this chapter. I will end up my introduction part with my aims.

1.1. Obesity

Obesity is increased adipose tissue due to unbalance between energy intake and expenditure and following weight gain (Wali, Thomas, & Sutherland, 2014). The prevalence of obesity in both women and men has increased worldwide in last decades. People are getting fatter in time because of transitions in their life styles and feeding habits. According to World Health Organization (WHO), it is estimated that 33.3% of women and 35.3% of men are obese in the world. The prevalence of obesity in children and adolescent is alarming. The level of childhood obesity is growing in time. According to National Health and Nutrition Examination Survey (NHANES), 16.3% of between 2-19 age of children and adolescent are obese. Regarding Nutrition and Health 2010 Reports in Turkey, prevalence of obesity is around %30 in male and female of general population. Moreover, diabetes has been observed on %19 of obese group. These numbers tend to increase without taking social precaution and doing scientific researches against obesity and diabetes.

Obesity is described as excess storage of energy as fat into adipose tissue in human (van Dam & Seidell, 2007). High nutrient intake and lack of physical activity lead obesity.

Obesity can be determined by physical parameters including bioelectric impedance analysis, skinfold thickness, underwater weighing, dilution methods and radiologic methods (Fields, Hunter, & Goran, 2000). These methods estimate fat composition into body. But, commonly body mass index (BMI) is the valid method to decide whether person is obese (Swinburn, Caterson, Seidell, & James, 2004). This

method is very practical and usable due to easy processing. BMI is calculated with this equation:

$$[BMI = Weight\ (kg) / Height\ (m^2)]$$

According to WHO, overweight is defined as BMI= 25.0-29.9 kg/m², obesity as BMI ≥30 kg/m². If the BMI value reaches 40 or more than 40, it is described as extreme or morbid obesity.

Function of adipose tissue can be differentiated regard storage location of fat. Fats are stored as visceral and subcutaneous in human. It has been indicated that excess fat storage as a visceral area brings high risk for obesity, type 2 diabetes (TD2), cardiovascular diseases (CVDs) than subcutaneous fat (Srinath Reddy & Katan, 2004). Genetic and nutritional factors are related development of the obesity (Vogler, Sorensen, Stunkard, Srinivasan, & Rao, 1995). Furthermore, age, gender, educational level, socio-cultural factors, psychological problems, economic level, genetic, hormonal and metabolic agents, the number of birth and the duration between births carry risk for obesity occurrence. These factors will be overviewed in detail later of this chapter.

1.2. The Health Problems Induced by Obesity

Obesity is one of the main contributors to progression of diseases including type 2 diabetes, cardiovascular problems, cancer, and hypertension. The studies have been clearly showed the link how obesity causes these health problems in human (Kahn, Hull, & Utzschneider, 2006). Beside of these serious problems, obesity might be also involved in endocrine, musculoskeletal, gastrointestinal, respiratory, and genitourinary problems.

Obesity is crucial factor for emergence of T2D. The majority of individuals with T2D is obese or overweight (Wing et al., 1987). This ratio is more than 80%, according to US data (Wali et al., 2014). Unbalanced glucose homeostasis, insulin resistance and increased hepatic glucose output in obesity causes T2D (Vogt, Domzig, Wabitsch, & Denzer, 2016). In normal condition, insulin can regulate carbohydrate and fat metabolism by inducing of glucose uptake from blood to skeletal muscles and adipose tissue. Insulin also provides inhibition of breaking down glycogen to glucose in the liver

(Wali et al., 2014). Moreover, insulin production leads molecular signals to inhibit catabolism of fats in adipose tissue (Tang et al., 2016). Overproduction of insulin from pancreas plays role on development of insulin resistance results in reducing glucose uptake in mainly adipose tissue and skeletal muscles (Alberti & Zimmet, 1998). The activation of insulin receptor by pancreas-derived insulin is impaired in T2D. At first of the obesity, pancreas might try to adapt high blood glucose level by increasing insulin production. This is protection mechanism to decrease blood glucose levels, but IR does not give response to insulin (Wali et al., 2014). In conclusion, obesity firstly induces to increase insulin resistance afterwards, causes T2D by various mechanisms (Kahn et al., 2006).

1.2.1. Cardiovascular Diseases

Coronary heart disease, cardiac insufficiency, and atherosclerosis have been observed in both obese women and men individuals (Poirier et al., 2006). Every 1-kilogram weight gain rises risk of coronary artery disease by 3.1% (Hubert, Feinleib, McNamara, & Castelli, 1983). In the same way, according to American Heart Association (AHA), a unit increment in BMI enhances congestive heart failure in men by 5%, in women by 7%. Obesity affects lipid profile in human and insulin resistance plays role in hyperleptinemia, hypertriglyceridemia, high LDL-cholesterol (low density lipoprotein), VLDL-cholesterol (very low density lipoprotein), and low HDL-cholesterol (high density lipoprotein) (Swinburn et al., 2004). When the amount of LDL-cholesterol increases, it generates plaques on the inner surface of the vessels by inducing inflammation and foam cell formation (Berg & Scherer, 2005). Growing plaque layer causes arteriosclerosis and following, it becomes the vessels are not capable carrying blood results in myocardial infarction and stroke (Poirier et al., 2006).

1.2.2. Cancer

According to National Cancer Institute, obesity has a relation with the esophagus, pancreas, breast, and gallbladder cancers. Obesity raises the risk of cancer due to creating hormonal and metabolic changes in the body (Key et al., 2004). Continuous and the big amount of secreted various hormones and growth factors from

adipocyte to the blood stimulates the cells to grow and divide faster than normal level (Sturgeon et al., 2016). This condition can trigger carcinogenesis. For instance, adipose tissue secreted high amount estrogen in obese people, this relates with developing risk of endometrial and breast cancer. Moreover, leptin is another hormone which is secreted from adipose tissue. Excess leptin can be associated with high-level cell proliferation in esophagus and colon tissue and causes cancer in these tissue (Tutino, Notarnicola, Guerra, Lorusso, & Caruso, 2011). Enhanced insulin and insulin-like growth factor-1 (IGF-1) also may develop cancer risk by increasing cells dividing and proliferation rates (Hawsawi, El-Gendy, Twelves, Speirs, & Beattie, 2013).

1.2.3. Hypertension

Hypertension is defined as limit exceeding of 140/90 systole and diastole blood pressure and one of the main risk factors of cardiovascular diseases particularly, heart attack (Srinath Reddy & Katan, 2004). According to Framingham Heart Study, obesity is associated with hypertension in men by 78%, in women by 65% (Wolf et al., 2007). Hypertension is correlated with bad lipid profile in human blood (Soran, Schofield, Adam, & Durrington, 2016). High level of cholesterol and triglycerides, high LDL-cholesterol or low HDL-cholesterol enhance risk of hypertension (Brown et al., 2000). In addition, systemic inflammatory state in obesity is associated with high blood pressure. One class of immune cytokine, interleukin-6 (IL-6), is interrelated with elevated systolic and diastolic blood pressures (Sowers, Epstein, & Frohlich, 2001). Moreover, it has been known inflammatory cytokine, IL-6, has relation with obesity and insulin resistance (Wali et al., 2014). So it is recommended that obese people need to decrease their blood pressures by losing body weight.

1.3. The Main Risk Factors that Leads to Obesity

1.3.1. Genetic Factors

Obesity is a disorder that affected from behavioral, environmental, and genetic factors (Bouchard, 2001). These factors can affect each other and lead to development of the obesity. Genes can change the sensibility of obesity and also excess food intake

with inadequate physical activity are directly related to obesity (Cummings & Schwartz, 2003). The genetic effect on obesity is explained with energy expenditure, appetite control, regulation of adipogenesis, lipid and carbohydrate metabolisms (Cummings & Schwartz, 2003). In addition, genetic factors are involved in controlling insulin sensitivity, cardiovascular risk factors, the loss in weight and the diet dependent lipid profile (Mutch & Clement, 2006). It has been suggested that more than 400 genes might be linked with obesity. Most studied genes on obesity are LEP (Leptin), LEPR (Leptin receptor), MC4R and MC3R (Melanocortin receptor), POMC (Pro-opiomelanocortin), PC1 (Proprotein convertase 1), PPARγ (Peroxisome proliferator-activated receptor), UPC1 (Uncoupling protein 1) (Clement, Boutin, & Froguel, 2002). Both genetic changes on these genes and external factors lead obesity or other metabolic diseases (Vogler et al., 1995). Lep protein is main controlling factor to regulate food intake. Leptin deficiency in animal model develops obesity (Clement et al., 2002). Overproduction of the Lep protein shows leptin resistance in obese human or human who consumes high fat or carbohydrate and exact mechanisms are still unknown (Campfield, Smith, Guisez, Devos, & Burn, 1995).

1.3.2. Hormonal Factors

Adipose tissue is the biggest source of energy in the body. Adipocytes secretes hormones that have role on body weight balance, immune system, blood circulation and steroid metabolism (Fain, Madan, Hiler, Cheema, & Bahouth, 2004). Production of the hormones from adipose tissue is correlated with fat mass into body (Hershkop et al., 2016). These hormones are called as adipokines that are hallmark of the obesity and T2D. In this part, main adipokines will be introduced with their function and role in obesity.

1.3.2.1. Resistin

Resistin is polypeptide hormone that secreted from adipose tissue and has a link obesity and T2D (Klebanova, Balabolkin, & Kreminskaia, 2007). According to recent studies, diet induced obese and T2D rat model shows elevated resistin levels (Kusminski, McTernan, & Kumar, 2005). In a way, resistin works as antagonist of

insulin on glucose metabolism (Beltowski, 2003). Therefore, it is called resistin for resistance to insulin. Resistin decreases glucose tolerance and enhances production of hepatic glucose in obesity (Steppan et al., 2001). According to *in vivo* studies, when resistin antibodies were given to dietary obese rats, it raises the insulin sensibility by fixing insulin resistance and hyperglycemia (Kusminski et al., 2005). It has been also observed that a type of anti-diabetic drugs, thiazolidinedione (TZD), causes down-regulation of resistin mRNA level (Steppan et al., 2001).

1.3.2.2. Tumor Necrosis Factor alpha (TNFα)

TNFα is an inflammatory cytokine and secreted from adipose tissue (Nascimento et al., 2016). TNFα controls volume and amount of fat in adipocytes via binding to TNFα receptor (Lee et al., 2016). Obesity also enhances synthesis of TNFα receptors in adipose tissue and this results in decreasing the number of insulin receptors (Kahn et al., 2006). Besides, TNFα inhibits the effect of insulin on adipose and muscle tissue. Thus, blocks glucose uptake to these tissues (Beltowski, 2003). The other function of TNFα decreases expression of free fatty acid transporters in adipocyte cells results in elevated fatty acid in blood and consequently hyperlipidemia is observed in obesity (Hotamisligil & Spiegelman, 1994).

1.3.2.3. IL-6

It is another inflammatory cytokine that is secreted by many tissue cells including T/B cells, fibroblast, endocrine cells, myocyte, endothelium, and intestine (Fain et al., 2004). It is observed that the level of IL-6 is associated with abnormal adiposity, disorders in lipid metabolism, T2D (Nascimento et al., 2016). These indicates effect of IL-6 on obesity. In obese people, visceral fat which is more related to progression of obesity, produces 3 fold more IL-6 than subcutaneous fat (Hershkop et al., 2016).

1.3.2.4. Adiponectin

Adiponectin is a plasma protein that secreted from adipose tissue. It is observed that the level of plasma adiponectin is low in patients with obesity, T2D and cardiovascular disease (Kusminski et al., 2005). Unlike the other proteins that were mentioned above, adiponectin reduces insulin resistance in many tissue and decreases the amount of free fatty acid in blood (Beltowski, 2003). Effect of adiponectin on insulin resistance enhances glucose uptake into peripheral tissues (Lis, Pilarski, & Bogdanski, 2015).

1.3.2.5. Leptin

Leptin is a hormone that have 167 amino acids and coded by LEP gene, also known as *ob* gene (Chung et al., 1998). Leptin is produced constitutively by adipose tissue also, by stomach, placenta, breast tissue, skeletal muscle, ovary and heart (Klok, Jakobsdottir, & Drent, 2007). Insulin, infections, glucocorticoids, overeating stimulate leptin releasing from adipose tissue whereas thyroid hormone, growth hormone, free fatty acids, long term hunger suppress leptin releasing (Considine et al., 1996). The functions of leptin are regulated by food intake, empty stomach and energy balance (Gura, 2000). When leptin is released into the blood, it passes through blood-brain barrier and reaches to hypothalamus to regulate its receptor on plasma membrane (Chung et al., 1998). Besides, the level of plasma leptin is directly associated with the amount of body fat, daily food intake, energy expenditure and hyperinsulinemia (Friedman, 2000). It was indicated that the leptin level is 10 ng/mL in the individuals with low body weight, 10-30 ng/mL in normal and more than 30 ng/mL in obese people groups. Its level shows increment by 40% after food intake (Considine et al., 1996).

The appetite control of leptin is ensured by giving satiety signal via leptin receptors (LEPR) (Chung et al., 1998). According to studies, the mice which have mutant *ob* gene (ob/ob mice) shows obesity characteristics due to absence of satiety signal (Campfield et al., 1995). In obese people, it is observed that a failure in leptin signaling or a resistance to leptin effect causes increased plasma leptin level (Gura, 2000). Leptin resistance is similar as insulin resistance, but mechanisms are still unknown (Enriori, Evans, Sinnayah, & Cowley, 2006).

LEPR that is mainly located in hypothalamus is activated by leptin. In healthy condition, when leptin binds to LEPR, it activates signal transducer and activator of transcription 3 (STAT3) and STAT3 goes into the nucleus (Tartaglia et al., 1995). It stimulates gene expressions that play role on appetite, physical activity, metabolic rate, energy storage and growth (Campfield et al., 1995). Furthermore, LEPR activates phosphoinositide 3-kinase (PI3K) pathway which is also activated as well as insulin signaling (Tartaglia et al., 1995). The body tries to regulate its energy balance and fat levels via PI3K. Mechanism that involves in leptin resistance occurs feedback mechanism. If tissues are exposed high level of leptin with long periods, cytokine signaling 3 (SOCS3) protein is secreted from liver and brain (Yang & Barouch, 2007). SOCS3 inhibits to STAT3 pathway results in low level of leptin production from adipose tissue (Considine et al., 1996). Another protein, protein-tyrosine phosphatase 1B (PTP1B), plays role on the negative regulation of leptin signaling through STAT3 pathway (Gura, 2000). These cellular mechanisms can decrease the ability of leptin and change the activity of neurons that stimulate physiologic effects of leptin.

1.3.3. Nutritional Factors

Carbohydrates are one of the most common organic molecules in the nature. Dietary carbohydrates can be reduced monomer to absorption through small intestine of human. Glucose is common monomer (found at least 50%) on disaccharides and main contributor for obesity and diabetes (Alberti & Zimmet, 1998). Glucose mainly used a sweetener in food products that are cheap, ready to eat, and very common on market. These factors increase glucose containing food intake all age groups.

Glucose is principle constituent of carbohydrates and a six-carbon monosaccharide. It is also known as blood sugar. The normal level of glucose in the blood is 70-100 mg/dl. If the level is lower than this limit, it is called as hypoglycemia, if the level is higher than the limit, it is called as hyperglycemia. Also they are primary energy source of body more specifically for brain. This project is subjected glucose in intestine so glucose metabolism and its relation on diseases will be introduced.

1.4. Digestion

Carbohydrate digestion begins in mouth by salivary amylase that breaks the long chain polysaccharides into shorter polysaccharide chains then partially digested carbohydrates, chyme reaches to stomach where carbohydrate digestion is stopped due to low pH. All the complex carbohydrates (polysaccharides, disaccharides, etc.) must be reduced to monosaccharides for intestinal absorption. Thus, carbohydrate digestion is completed in small intestine. Small intestine consists of three parts, duodenum, jejunum and ileum. Carbohydrate digestion mainly occurs in duodenum and upper jejunum (Wright, Hirayama, & Loo, 2007). When chyme comes to duodenum, pancreatic amylase is secreted by stimulated pancreas to break down polysaccharides into disaccharides. Thereafter, disaccharides (maltose, lactose, sucrose) are broken down into monosaccharides (glucose, galactose, and fructose) by the enzymes including maltase, sucrose, lactase that are produced by enterocyte cells of small intestine. Maltose is broken down into two glucose molecules, sucrose; glucose and fructose, lactose; glucose and galactose (Dashty, 2013). When disaccharides are hydrolyzed, at least half of released molecules are glucoses. Consequently, the complex nutrients are become monomer forms that are able to transport into the blood.

1.5. Absorption

Enterocyte cells are main cell type that is responsible for carbohydrate absorption. Enterocytes shows polarized cell morphology. Apical side refers place where dietary nutrient are taken up into cells and basolateral side release absorb nutrients through blood (Mahraoui et al., 1994). Carbohydrates only can be transported via enterocytes while they are monomer (glucose, fructose and galactose). Glucose molecules are absorbed into the body by specialized transport proteins that are located in the apical and basolateral sides of enterocytes.

It is indicated that there are two main glucose transporter groups in human intestine. These two groups are different from each other in terms of their mechanisms. The first group is the members of glucose transport (GLUT) proteins that transport glucose from high concentration medium to low concentration medium. Primarily tissues and cells that GLUT proteins express are, for GLUT1; placenta, liver, kidney

and colon, GLUT2; liver, pancreas β-cells and small intestine, GLUT3; brain and testis, GLUT4; skeleton, cardiac muscle and adiposity, GLUT5; small intestine and sperm. Only GLUT4 shows an insulin-dependent regulation on adipose and muscle tissues (Leto & Saltiel, 2012). GLUT2 ensures to give the glucose from enterocyte to the blood and then glucose is utilized all over the tissues.

The members of the second group are sodium-dependent glucose cotransporter 1 and 2 (SGLT1 and SGLT2). SGLT proteins transport glucose from low concentration medium to high concentration medium. The amount of intracellular sodium is important for glucose uptake due to being sodium-dependent manner (Grefner, Gromova, Gruzdkov, & Komissarchik, 2014). SGLT1 is expressed in apical side of enterocytes. GLUT and SGLT proteins functions together to provide fuel for energy. Activity of SGLT1 is dependent intracellular sodium and potassium ions (Roder et al., 2014). Sodium/potassium adenosine triphosphatase (Na^+/K^+-ATPase) protein on basolateral side of enterocyte control Na/K ratio in enterocytes. When intracellular Na ion is decreased, this triggers Na ion absorption by SGLT1. So, two sodium molecules enter into the cell in response to each taken glucose (Shirazi-Beechey, 1995). Na^+/K^+-ATPase hydrolyzes adenosine triphosphate (ATP) and this energy is utilized to take two molecules potassium into cells and three sodium molecules are out of the cells. Balance of Na/K is essential on intestinal glucose absorption. Enterocyte glucose absorption was illustrated with detail on figure 1.1.

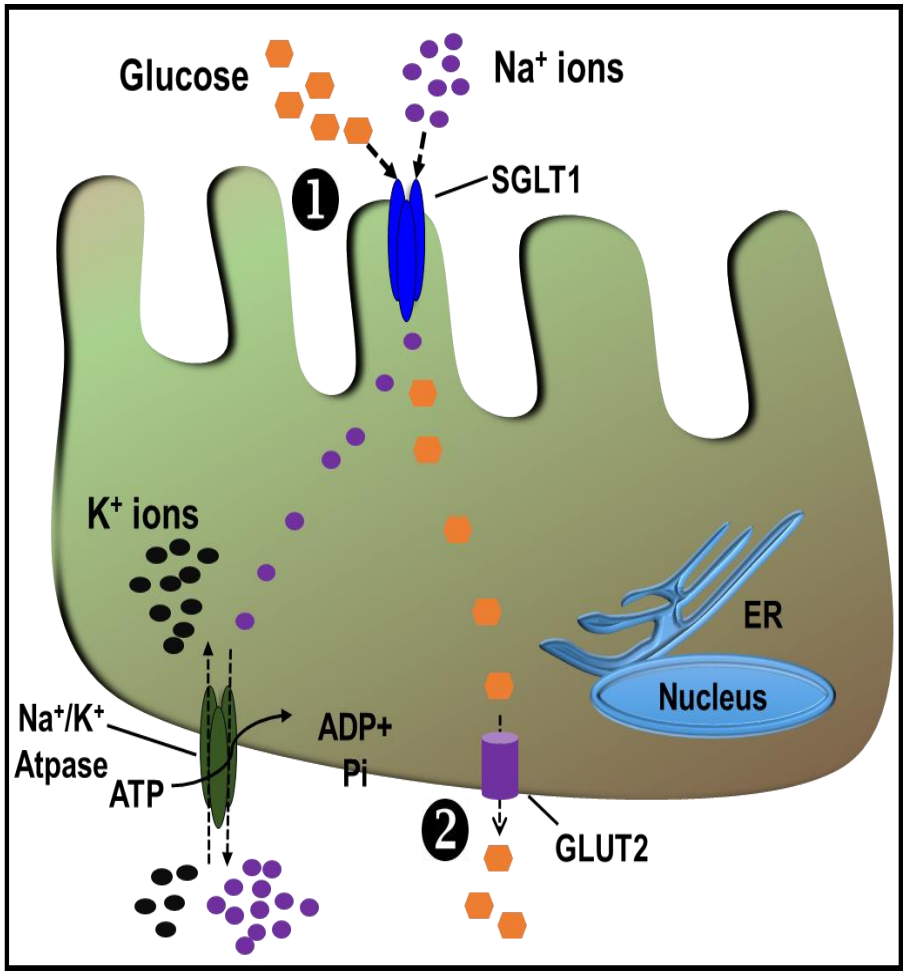

Figure 1.1. Glucose absorption of enterocyte. Two pathways are shown. (1) SGLT1 transport protein is located in apical side of enterocyte and responsible from glucose uptake into the enterocyte. It is dependent to Na^+/K^+ ATPase transport protein. Together ensure the balance of intracellular Na^+ ions. (2) GLUT2 transport protein is located in basolateral side of enterocyte and its function is giving absorbed glucose to the blood.

1.6. Molecular Relation Between Glucose, Insulin Signaling and Obesity

Carbohydrates primary factor that cause to increase insulin releasing. After absorption of carbohydrates through enterocyte, glucose passes into the bloodstream and its level is elevated. This is signal for pancreases to release insulin from β-cells.

β-cells are the most glucose sensitive cell type in human (Chang, Chiang, & Saltiel, 2004). When insulin reaches to first adipose and muscle tissues, it binds to insulin receptor protein (IR). IR is a dimer transport protein that is composed of α-subunits and β-subunits. IR is a member of receptor tyrozine kinases indicates that phosphorylation/dephosphorylation regulates IR and downstream of IR pathways (Kahn et al., 2006). Insulin first binds to α-subunits and then induces conformational changes on β-subunits. This phosphorylates insulin receptor substrate I (IRS-1). After that, the phosphorylated IRS-1 binds to the enzyme phosphoinositide 3-kinase (PI-3K). The activation of PI-3K apprises of the conversion of PIP2 to PIP3. Following that, PIP3 binds to AKT. The activation of AKT induces the translocation of GLUT4 to plasma membrane, hence, glucose can be transported into the cell so, the blood glucose level decreases (Wali et al., 2014). GLUT4 appears in the cytoplasm and found in the form of vesicle in the absence of insulin. At the end of insulin stimulation, GLUT4 vesicle fuses with the plasma membrane and starts to glucose uptake (Chang et al., 2004).

Insulin increases glucose transport adipose and muscle cells. If these tissues have enough glucose to use as energy source, all excess glucose is converted to fatty acid. This is main contribution of glucose to development of obesity. Detail of this mechanism is that glucose is broken down to pyruvate by glycolysis, then, pyruvate generates acetyl co-A. This molecule is the substrate synthesis of fatty acids. When excessive amount of glucose used for energy, excess citrate and iso-citrate emerge and directly activate acetyl co-A carboxylase enzyme that needs to start to the first step of fatty acid synthesis. Glucose derived fatty acids can be stored in adipose or muscle cells or is transported liver to be stored as fat.

When the cells are exposed with excess fatty acids, GPR120 is a member of family of G-protein coupled receptor can be regulated in adipose tissue. Activated GPR120 sends signals to stimulate PI3K/AKT and then promotes glucose uptake (Chang et al., 2004). Another G-protein coupled receptor is GPR40, also known as free

fatty acid receptor 1 (FFA1). GPR40 is expressed in pancreatic β-cells. When the level of blood glucose increases, also the gene expression of GPR40 increases. Thus, GPR40 may play a role to initiate the secretion of insulin (Yamada et al., 2016). In obesity, the amount of glucose and insulin rise (Bagdade, Bierman, & Porte, 1967). The relation between glucose and obesity has two aspects. The first aspect is that glucose causes weight gain in the body because glucose can be stored as fat that detail mechanism was explained earlier of this chapter. The second relation between obesity and glucose is that obesity influence the glucose metabolism and induces diabetes by revealing insulin resistance. However, the molecular mechanism of this relation is unknown (Bloomgarden, 2008).

Diabetes has two types in the body; type 1 diabetes and type 2 diabetes. Type 1 diabetes (T1D) is an autoimmune disease that T-cells attack pancreatic β-cells and impair their functions (Daneman, 2006). Production of insulin is diminished from β-cells. Thus, GLUT4 that is insulin-dependent transport protein is not able to function and consequently, the glucose in the blood cannot be taken sufficient amount from blood to fat and muscle tissues (Rea & James, 1997). Individuals with T1D must be injected by insulin daily. Obesity is the primary cause of T2D. In this situation, although insulin is produced by β-cells, insulin-dependent glucose uptake in muscle and fat tissue does not occur. This causes the existence of more glucose in the blood. The known molecular mechanism of T2D occurrence is extreme phosphorylation of insulin receptor results in insulin resistance (Pederson, Kramer, & Rondinone, 2001). Another aspect is that the activation of obesity related c-Jun N-terminal kinase 1 (JNK1) augments and insulin resistance is observed in fat, muscle and liver tissues (Hirosumi et al., 2002). There are still unanswered questions for progression of the TD2 during obesity.

1.7. Enterocytes and Nutrient Sensing

Small intestine consists of specialized and differentiated cells including enterocyte, goblet, Paneth and endocrine. Goblet cells are responsible for secretion of mucosa on the villus of the intestine. These cells create mucosa barrier to protect body from infection and other harmful substances (Specian & Oliver, 1991). Paneth cells produce antimicrobial molecules like neutrophils that protect small intestine against to microorganisms (Ouellette, 1997). Endocrine cells play role in production and excretion

of hormones and regulate the metabolic activities (Cheng & Leblond, 1974). Enterocyte cells cover all over intestine villus and main function is to absorb nutrients (Shirazi-Beechey, 1995). These cells are the first cell group that contact with nutrients and constitute 90% of all cell population of the intestine (Shirazi-Beechey, Moran, Bravo, & Al-Rammahi, 2011). These cells are polarized and can renew themselves every 48-72 hours. Apical side of the enterocytes can recognize specific dietary nutrients via receptor and transport proteins (Vereecke, Beyaert, & van Loo, 2011). Thus, nutrients dependent regulation of the enterocytes is important for human physiology.

Enterocytes are the first cell group that contact with glucose and also, responsible for their absorptions. These properties of the enterocytes ensure to play active role in glucose uptake in a controlled manner. However, it has not known how elevated glucose levels affects to gene regulations and intracellular molecular mechanism inside of the enterocytes cells (Mahraoui et al., 1994).

The level and rate of the glucose absorption from enterocyte cells are important in obesity and diabetes. It has been shown that glucose uptake in enterocyte cells can be controlled pharmacologically by two mechanisms. First, the function of SGLT1 protein is decelerated by using SGLT1 antagonist (Tahrani, Barnett, & Bailey, 2013). Second, α-glucosidase enzyme inhibitors are used to reduce production of the carbohydrates monomers (Liu, Sim, Rose, & Pinto, 2006). Nevertheless, the efficiency of these medications and chemical molecules are still being discussed. For example, acarbose is an active substance that used in anti-diabetic drugs to inhibit glucosidase enzymes results in low glucose absorption through small intestine (Chen et al., 2016). It helps to reduce the blood sugar, but anti diabetic drugs have many side effects including hypoglycemia, diarrhea, failure in liver functions, stomach-ache, edema and nausea (Rosenstock et al., 1998). Decreasing SGLT1 function causes inhibition of Cl^{-}/HCO^{-3} protein and alteration in ion balance in enterocyte cells. These changes induce diarrhea (Meyer, Porta, Garavaglia, & Cremaschi, 2001). Furthermore, enterocyte intracellular signal pathways are influenced in SGLT1 inhibition (Jung et al., 2009). Therefore, it is better to understand enterocyte glucose metabolism in detail in terms of obesity and T2D.

Obesity may have effect on nutrient digestion and absorption from enterocytes (Dailey, 2014). Obesity may influence recognition of the nutrient levels or types by enterocytes of cells. This recognition mechanism might be related to intracellular molecular pathways or specific genes. It is proposed that enterocyte cells have own

control mechanism to detect existence of dietary glucose on the lumen via taste receptor 2 and 3 (T1R2 and T1R3) (Dyer, Salmon, Zibrik, & Shirazi-Beechey, 2005). T1R2 and T1R3 double knocked out mice model showed that these proteins did not play direct role on glucose sensing on enterocyte cells. This indicates that another unknown protein(s) might compensate this molecular mechanism in intestine (Shirazi-Beechey, Moran, Bravo, et al., 2011).

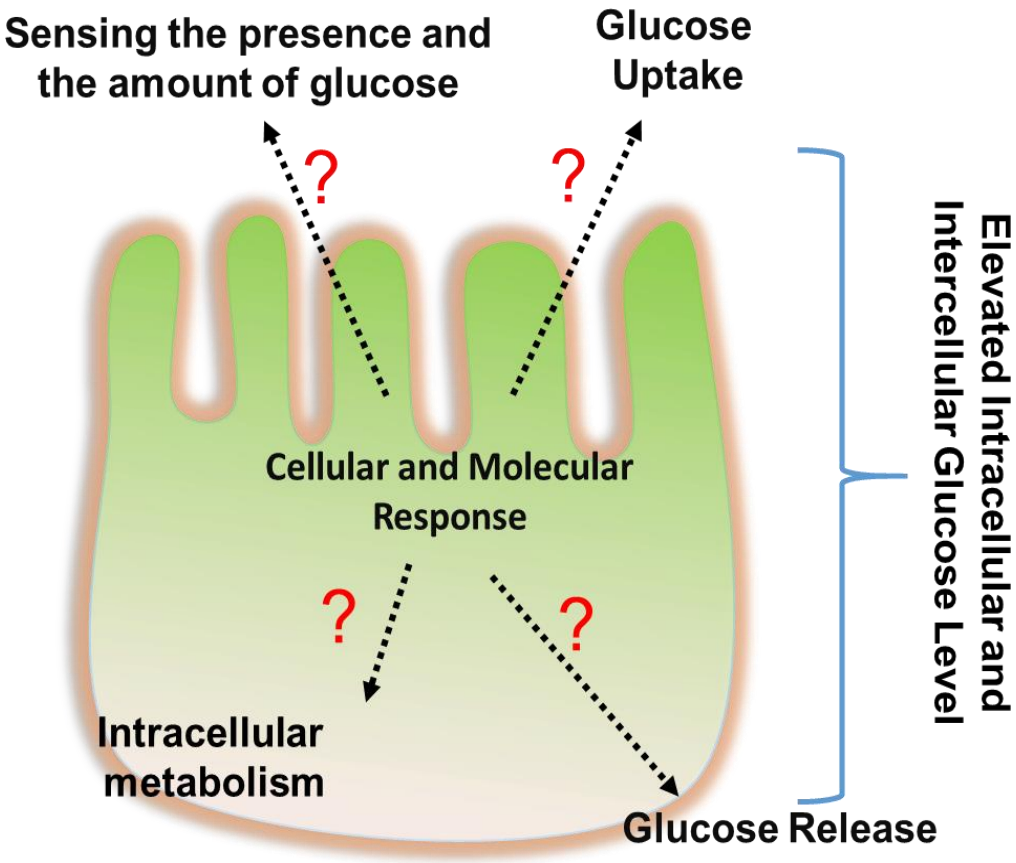

Figure 1.2. Indicates enterocytes specific unknown molecular and genetic response during high glucose condition.

In conclusion, consuming of the glucose from natural or fabricated food are tremendously increased in all over age's groups in the world. Glucose is one of the main dietary contributor to leads obesity and T2D. It was known that liver, adipose, pancreas, and brain tissue play a role on relation glucose and pathogenesis of the obesity. Moreover, dietary intake and blood glucose levels are elevated in obese human and animal models (Loh, Moy, Zaharan, Jalaludin, & Mohamed, 2016). Thus, enterocytes of the intestine cells are exposed high glucose molecules daily. However, it has not been investigated how enterocytes give molecular and genetic response to high glucose (summarized on Figure 1.2). To achieve this, two specific aims were pursued:

AIM I: To investigate how elevated glucose level affect *genome-wide molecular and genetic response* in fully differentiated human enterocyte model

AIM II: To determine which *metabolic pathways* affected in enterocytes by analyzing gene expression levels

CHAPTER 2

MATERIALS AND METHODS

2.1. Caco-2 Cell Culture Study

Direct using of differentiated noncancerous primary enterocyte cells from human and animal intestine tissue is difficult and they can be alive for very short time period as ex vivo (Rousset et al., 1985). Thus, the constitution of alternative models are necessary and very important for intestine specific approaches. Caco-2 cells are human colon cancer cell line that procured from a human colon adenocarcinoma. This cell culture model was defined first time in 1970 and has been used in cancer study models (Fogh, Fogh, & Orfeo, 1977). It was observed when Caco-2 cell culture model is fully differentiated on special membranes, they substantially resemble to enterocyte cells in human body with regard to features of metabolic, functional and biological activity (Sambuy et al., 2005). When we compared Caco-2 cell line with other type of colon carcinoma cell models, Caco-2 cell line has better morphological and functional structure for investigation of nutrient metabolism (Chantret, Barbat, Dussaulx, Brattain, & Zweibaum, 1988). Caco-2 cell line is the most common *in vitro* cell model that used in the absorption studies of nutrients (Yin et al., 2014), drugs (Wilson, 1990), functional molecules (Satake et al., 2002) and enterocyte mineral metabolism (Hu, Gulec, & Collins, 2010).

The other advantage of studying with this cells, when the cells grow up on special membrane and they can differentiate, furthermore can be polarized (Borchardt, 2011). When these cells grow for 21 days on special membrane in the optimum conditions, they perfectly mimic to human small intestine system (Sambuy et al., 2005). The polarized cells are specialized and modeled as apical side where is exposed with the nutrients and basolateral side where is contacted with blood vessels (Roder et al., 2014). Furthermore, Caco-2 cell line is frequently used in the glucose metabolism studies of small intestine. The proteins and other cellular factors, which play a role on glucose metabolism, are synthesized in this cell line (Sambuy et al., 2005). Thus, we utilized CaCo-2 cell line as *in vitro* model of human intestine in this proposal.

In our project, Caco-2 cell line (ATCC Number: HTB-37) were obtained ATCC cell culture collection. Dulbecco's Modified Eagle's Medium (DMEM) with 5,5mM glucose (Sigma cat no: D6046) and DMEM with 25mM glucose (Sigma cat no: D6429) were used as a growth basal medium. 15% MEM Fetal Bovine Serum (FBS) (Gibco cat no: 10500), 1% Gibco Penicillin-Streptomycin (Gibco cat no: 15140-122) and 1% MEM Non-essential Amino Acid Solution (100×) (Gibco cat no: 11140-050) were added into to make complete growth medium. The 10^6 cells in 10 mL growth medium were maintained in 100mm diameter petri dish and humidified atmosphere at 37 °C with 5% CO_2 in the cell culture incubator. When the cells reached 90% confluence, we divided cells with 1:5 ratios on new plates. As an experimental conditions DMEM with 5.5mM and with 25mM glucose were used control and high glucose conditions respectively. The stock CaCo-2 cells were prepared with cell freezing solution (5% DMSO and 95% DMEM medium (contains 20% FBS)) liquid nitrogen due to use when cells are needed. The passage number was kept between 20-30.

2.2. Modeling of Human Intestine System *In vitro*

To create in vitro human intestine system, CaCo-2 cells were grown on collagen coated inserts. The insert system was described on figure 2.1. Briefly polytetrafluorethylene (PTFE) (Corning cat no: 3493) membrane has 0.4 micron pore size inner and it can be placed on 12mm diameter cell petri dishes. At the first step of the experiment, 103 Caco-2 cells were seeded on the membrane. 700µl medium to apical sides and 1.400µl medium to basolateral sides were added. The growth of cells was observed regularly by the microscope. After 3 or 4 days, the cells covered the membrane. Thereafter, the cells need 21 days incubation to differentiate and polarized. Control DMEM medium was used until 10th day of the differentiation periods and then cells were divided two groups (Figure 2.3); first group was maintained same medium condition (DMEM with 5.5mM glucose) and second group was growth under high glucose condition (DMEM with 25mM glucose). The cells under two different groups were maintained for 11 days. Some experiments were done under different treatment strategies. When the cells were maintained on the 100 mm petri dishes, they were divided into two plates. The cells on the first plate was maintained control and second plate was under high glucose condition for at least three passages. After that the

cells transferred on membrane system that was explained above. The cells into two groups were maintained under earlier indicated glucose conditions for 21 days. Second glucose treatment strategies were used to create chronic high glucose treatment.

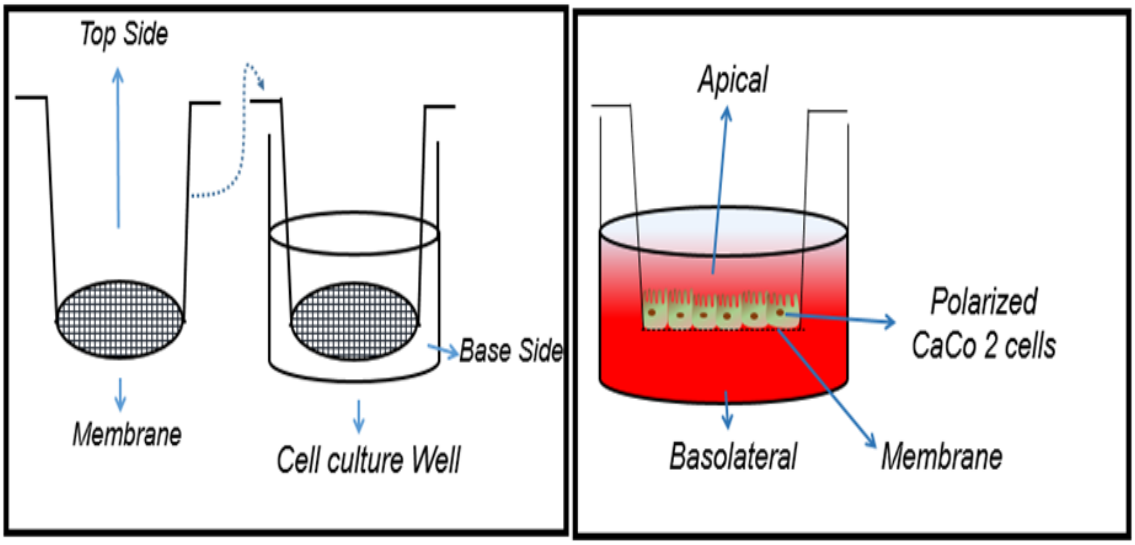

Figure 2.1. (A) Membrane and cell culture plate system and replacing of membrane into individual well, (B) after polarization of the cells to apical and basolateral sides on membrane.

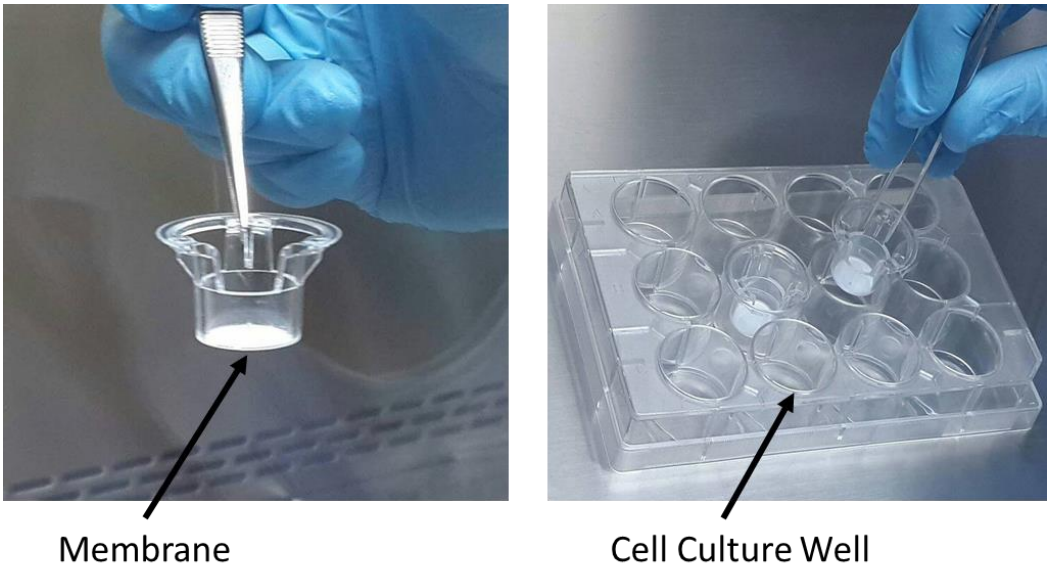

Figure 2.2. The real photos of membrane and the cell culture well were shown

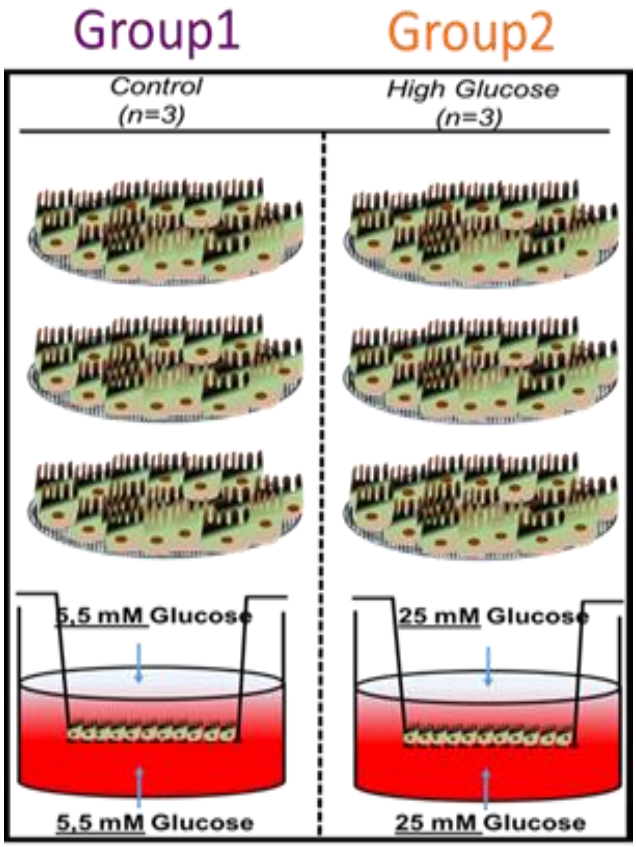

Figure 2.3. Experimental treatment conditions

2.3. Transepithelial Electrical Resistance (TEER)

When the cells constitute tight junction, polarized monolayer forms, they create a transepithelial electrical resistance barrier (TEER). TEER was used to check if the cells generate monolayer form and interact each other tightly. TEER value was measured in ohm by the EVOM device (WPI, USA) (Figure 2.4).

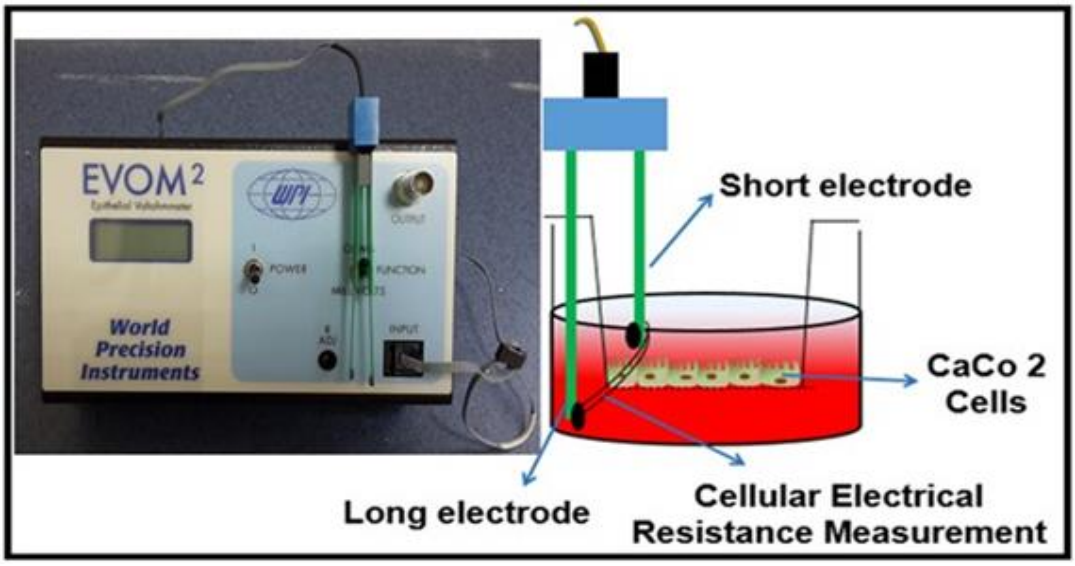

Figure 2.4. This figure shows how EVOM meter utilized to measure TEER on fully differentiated CaCo-2 cells

EVOM device has basically with short and long two different electrodes. The short electrode was inserted to apical side, the long electrode was inserted to basolateral side. When the cells show barrier resistance, this resistance is converted numbers per the cm^2 (Figure 2.4). This value is stated at least 250 ohm/cm^2 for Caco-2 cells (Liang, Chessic, & Yazdanian, 2000). Moreover, when the cells grow up to 21 days, TEER value is around 1000 ohm/cm^2. The meaning of the resistance is more than 250 ohm/cm^2 on cells, absorbable molecules go from basolateral side to apical side only via the cells. Therefore, before continued further experiments, appreciated value of the TEER is essential. TEERs were measured for the cells that used at experimental stage at 21^{st} day of growth then further experiments were done.

2.4. Measurement of Basolateral Glucose

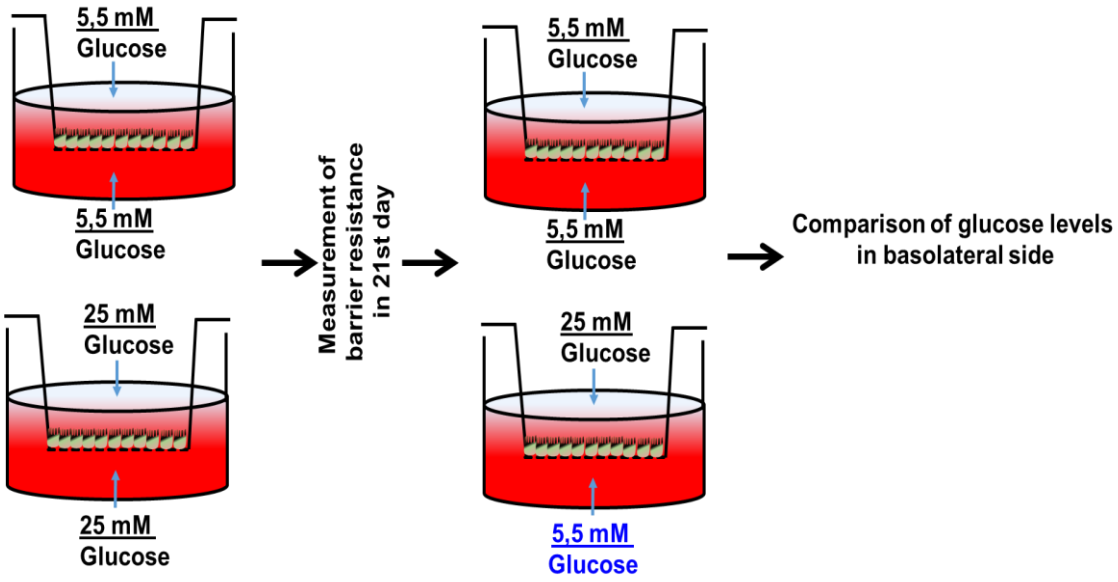

Figure 2.5. Experimental strategy to measure glucose levels from basolateral side if the cells.

Glucose absorption in human occurs in small intestine and basic mechanism is that glucose is taken up from apical side to basolateral side of the intestinal cells and then glucose molecules go through other tissues. To test whether same situation occurred in our *in vitro* system we measured glucose levels as described on figure 2.5. All these measurement was begun after TEERs were reached at least 600 ohm/cm^2. We used two different methods to measure basolateral relative glucose levels from the two different experimental conditions at the different time points. We used classical glucose oxidase method to measure glucose levels (Nayak & Herman, 1997) and also to confirm our results second method was used to measure glucose levels by reducing sugar measurement by Nelson-Somogyi method. At 500 nm, Varian Cary Bio 100 UV-Visible spectrophotometer was used to read absorbance.

2.5. RNA Isolation

CaCo-2 cells that were grown on membrane were washed with classical PBS buffer and they were collated into tubes. Total RNA isolation was performed by RNAzol reagent (MRC, USA) regard to company protocol. Briefly, 1mL RNAzol was added to cells and cells were lysed by making vortex. 400 µL ultra-pure water was added for every 1mL RNAzol added cells. The samples were centrifuged at 12,000 X g for 15 min. around 80% of the supernatant was taken into new sterile tubes. 400 µL 75% ethanol was added to supernatant and then centrifugation at 12,000 X g for 8 min. After this step, the samples were washed two times more with ethanol. The next, ethanol was removed using micropipette and Diethylpyrocarbonate (DEPC) water (prepared with 1mL DEPC for 1000 mL ultra-pure water) (Omega bio-tek, E.Z.N.A.® Cat. no: R6934-00) was added to dissolve RNA sample pellets. The total RNA concentration was measured by Nano drop (Thermofisher, USA) instrument.

2.6. Determination of RNA Samples Quality

During isolation step, RNA samples can be affected from laboratory conditions such as temperature, sterilization and experience. Thus, the quality of the total RNA is important for downstream experimental steps. To test quality of the RNA samples, total RNA samples from two different groups were pulled and run on classical 1% agarose gel method. First RNA samples were incubated at 60°C for 3 minutes and agarose gel running was performed. RNA samples quality was determined by looking at the apparition of 28S and 18S fragments in the gel. If RNA bands were not seen or became weathered, this would show that there might be a problem at isolation steps.

2.7. cDNA Synthesis and Quantitative RT-PCR

mRNA samples were converted to complementary RNA (cDNA) synthesized by cDNA synthesis kit and regarding company protocol (Lifetech, USA. Cat no: 4368814). 1µg of the cDNA was used to perform Real Time Quantitative Reverse Transcription (RT-qPCR) method. Classical SYBR® Green method was used by ready to use fabricated kit (Lifetech, USA. Cat no: 4367659). Relative mRNA expression levels

were determined ABI-stepOneplus RT-qPCR equipment (Lifetech, USA). Gene specific primers were selected from other studies. Mean fold changes of mRNA were calculated by using the $2^{-\Delta\Delta Ct}$ analysis method as previously described (Gulec & Collins, 2014).

2.8. Bioanalyzer

The Agilent 2100 Bioanalyzer was utilized for checking the quality of RNA samples. This system performs small-scale gel eletrophoretic separation of RNA by using a microfluidic chip. RNA 6000 Nano reagents was used. Bioanalyzer has many advantages which are quick separation and minimal sample volume (1µl) is required (Davies, Denyer, & Hadfield, 2016).

2.9. Determination of mRNA Expression Profile and Gene Ontology Analysis

50 bases probs are used for every specific gene in analysis beadchips of whole genome gene expression. The used beadchips (Figure 3.11) have the beads that coded on them and purified cRNA can be hybridized on. cRNA samples that purified from total mRNA directly hybridizing to beads on the beadchips. There are apart canals on a beadchip to work with 12 samples. Every each canal has around 48.000 different kind of beads all scattered throughout the genome.

First step starts with labeling samples which is called as cRNA purification. Ambion Illumina TotalPrep RNA Amplification Kit was used in this application. This kit works with between 50-500 ng isolated total mRNA. cDNA synthesis was performed according to kit protocol. The other steps follows this step in order of second strand cDNA synthesis, cDNA purification, in vitro transcription to synthesize cRNA and cRNA purification. Second step is the measurement of cRNA concentrations. This stage was performed by Nanodrop as spectrophotometric device. Essential total cRNA concentration for microarray is 750 ng. Third step is microarray beadchip application. At the same time 12 samples can be worked on a beadchip surface. After loading the samples to the chip, beadchip is left overnight for hybridization and next washed with

solutions. The following washing, the beadchip is labeled with streptavidin and then the beadchip is read and analyzed by microarray laser reader.

Figure 2.6. HumanHT-12 v4 Expression BeadChip
(Source: Eurofins Genomics, 2016)

2.10. Statistical Analysis

The results were expressed as mean ± SE. All analyses were performed and figures were made in GraphPad Prism (version 6.0 for Windows, GraphPad). Relative gene expression were analyzed by student-t test.

The results of control group were normalized to 1 and the changes of high glucose group were given as fold change.

The pathways were designed by using online analysis tools (https://david.ncifcrf.gov).

Table 2. Primer list

Gene Symbol	Forward (5'-3')	Reverse (5'-3')	Reference (PMID)
ABCA1	GCACTGAGGAAGATGCTGAAA	AGTTCCTGGAAGGTCTTGTTCAC	11719471
ALDH2	CACTTCGCCCTGTTCTTCAACC	CCTGCTCGGTCTTGCTATCAAAG	21593812
ALDH6A1	GTGCTTCTGGGCAGTAGAG	TCACCTTGGAAGAAACCTGC	25099943
ALDOA	TCACCGCATCGTGGCACCTG	GAAGCGCCGGTTCTCCTCGG	25932951
GLUT2	CTCTCCTTGCTCCTCCTCCT	TTGGGAGTCCTGTCAATTCC	25010715
GLUT5	TCTCCTTGCAAACGTAGATGG	GAAGAAGGGCAGCAGAAGG	24426192
IGF2	CGAGAGGGACGTGTCGACC	GGACTGCTTCCAGGTGTCATA	24454871
INPPL1	TCGTCACCAGCGACCATTC	AGCCCTTTCTTGGAGATGAACTG	25525286
IRX	CTCTCCCTGCTGGGCTCT	CAAGGCACTACAGCGATCTG	25512384
PFKP	GCATGGGTATCTACGTGGGG	CTCTGCGATGTTTGAGCCTC	25932951
PFKFB3	CTGGACAGGGAGGGAGATACTA	AATGAAGAGCTTTGCCCGTGGTC	16115917
PGAM1	AGGTCACTGCCTACTGCCTG	ACATCACCACGCAGGTTACAT	25932951
PGD	TTATTTGTGGGGAGCGGAGT	TCTTTGTTCCCTCCTGGCAT	26147000
PRKCZ	GGGGGACATCTTCATCA	CTCGGGAAAACATGAATG	24990612
PYGL	CACTTCAGTGGCAGATGTGGTG	GCAGTGGAAATCTGCTCTGACAG	25001192
SGLT1	TGGCAATCACTGCCCTTTA	TGCAAGGTGTCCGTGTAAAT	24426192
SOCS2	GAGCTCGGTCAGACAGGATG	AGTTGGTCCAGCTGATGTTTT	22291912
TXNIP	ACAGAAAAGGATTCTGTGAAGGTGAT	GCCATTGGCAAGGTAAGTGTG	18171713
CYPA	TACGGGTCCTGGCATCTTG	CGAGTTGTCCACAGTCAGCA	(*)
LCN15	GCATCTGACTGCAGGGTCTT	CCTTCAGGTACTCGGCATCC	(*)

(*) The primers were designed by lab. members

CHAPTER 3

RESULTS AND DISCUSSIONS

3.1. CaCo-2 Cells

10.000 cells were put on the upper side of insert. Figure 3.1.A shows when the cells were seeded on membrane. They started to grow and became confluent in 3 or 4 days. Figure 3.1.B indicates that the cells were covered and differentiated all over the membrane after 21st days post-confluent and darker areas, dome, are indirect indicator of the differentiated cells on transwell systems (Ferraretto et al., 2007). The behavior of the cells on membrane was closely control every 2-3 days periods. Any detached cells on the membrane were excluded from experimental plates.

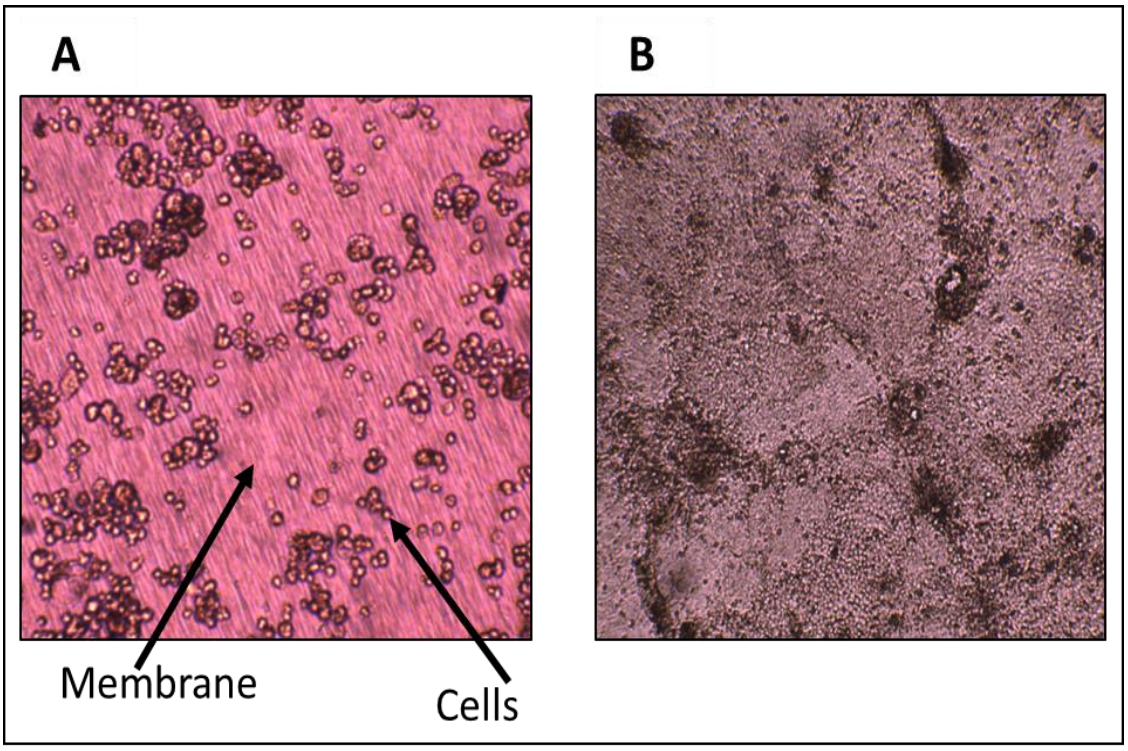

Figure 3.1. Microscopic photos of the cells. (A) shows 1st day on the membrane (4X), (B) shows 21st day on the membrane (10X)

3.2. Determination of TEER Values in Time

Even if we see dome features of the cells, we must confirm monolayer and polarized structures from cells by measuring transepithelial resistance (TEER). Different polarized cells have different barrier resistance on membranes. The TEER for CaCo-2 cell has been established in elsewhere (D'Souza, Shertzer, Menon, & Pauletti, 2003). Minimum TEER value for CaCo-2 is 250 ohm/cm^2. In 9th day of the post-confluence, the barrier resistance of cells reached to 250 ohm/cm^2. In 21st day, the value was measured around 1000 ohm/cm^2 (Figure 3.2). These measurements indicated that CaCo-2 cells were polarized and had monolayer structure with closed tight junctions. TEER values were measured from experimental cells before RNA isolation and downstream steps. Whenever we observed TEER values were lower than 250 ohm/cm^2, we excluded those wells from our experiments.

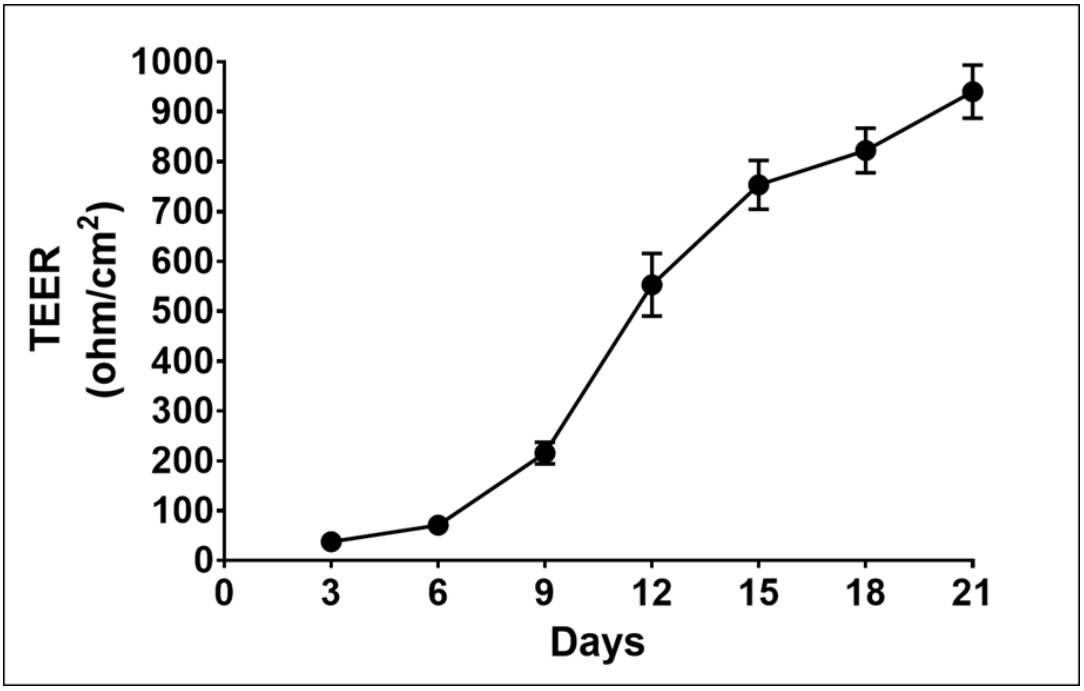

Figure 3.2. TEER values during post-confluence

3.3. Results of Basolateral Glucose Measurement

To test functional features of the minimalistic *in vitro* human intestine system, we looked at vectorial glucose transport capacity of control and glucose treated cells. This gave us information whether our experimental model worked as human intestine system in terms of dietary glucose uptake into enterocyte cells and efflux of glucose from cells to blood. Control and high glucose treated CaCo-2 cells were grown on the membrane for 21 days. TEER values were determined from control and glucose treated CaCo-2 cells. It was observed that there was trend to decrease for TEER from glucose treated cells, but it did not reached significance (Figure 3.3). The studies showed that high glucose might affect tight junction formation due to changing osmolarity of the cell culture medium (D'Souza et al., 2003). In that study 25mM glucose was arranged by adding glucose into medium and this might affect osmolarity of the medium. For this reason, we used two different cell culture mediums that contained 5.5mM and 25mM glucose concentration and other ingredients of the mediums were identical. Their osmolarity were set for physiological levels in these cell culture mediums. This limited osmolarity effect on monolayer CaCo-2 cells.

After 21 days cells mediums were replaced as indicated on figure 2.5 (at the material method part). We used classical glucose oxidase method to measure glucose levels (Collison et al., 1999). Medium from basolateral site were collected in the different time points. We found that glucose efflux was significantly increased between 90^{th} to 240^{th} time periods when glucose was given only apical sites with 25mM concentration (Figure 3.4). We initiated another glucose measurement method to confirm first measurement results, we selected most significant glucose efflux time point (240^{th} minute, $p \leq 0.0049$) and classical Nelson-Somogyi method was performed in samples from 240^{th} glucose incubation time point. We found very similar result that was observed from glucose oxidase method (Figure 3.5). These results shows that our experimental model behaves as similar to human intestine functions which are absorption of the glucose from enterocyte (into CaCo-2 cells) to blood (to basolateral site of the transwell system).

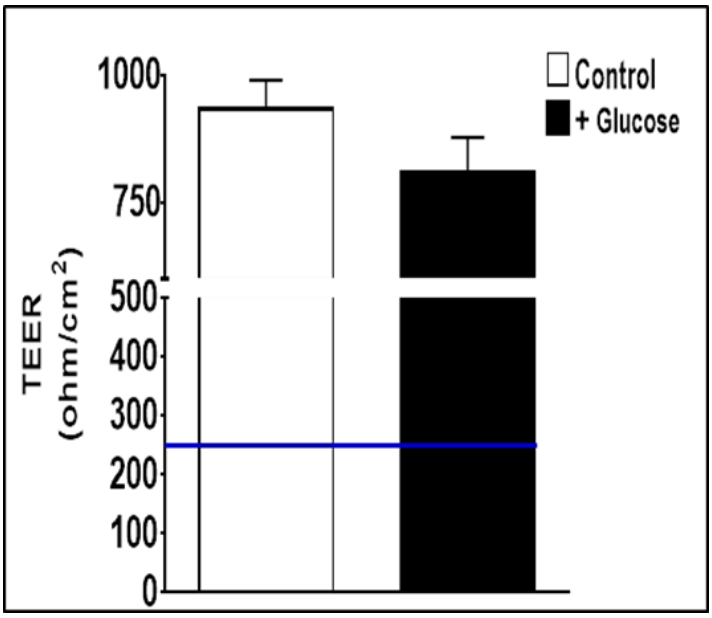

Figure 3.3. The level of barrier resistance of cells at the 21st day post-confluent

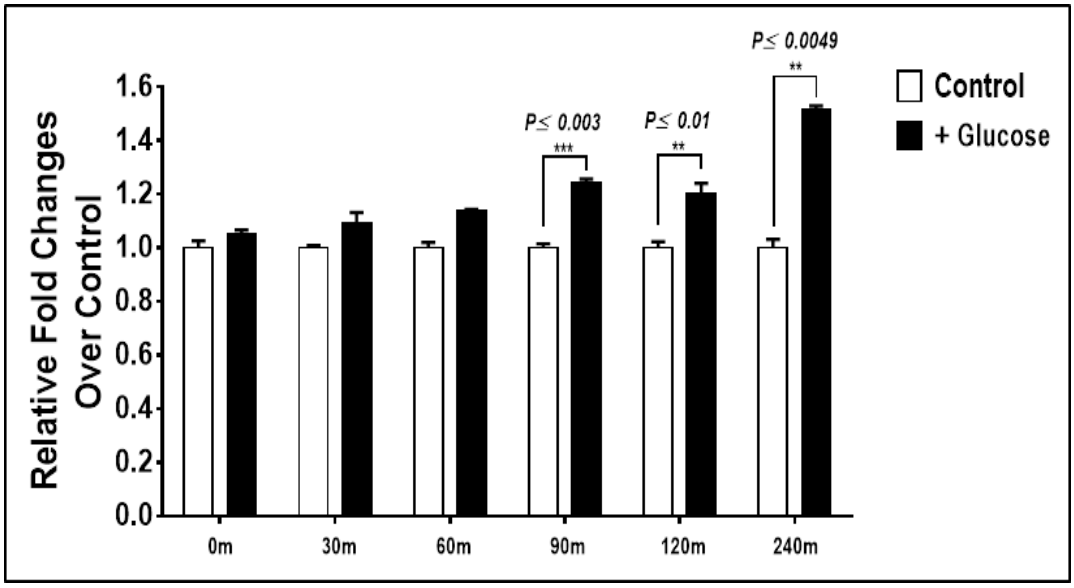

Figure 3.4. Relative fold changes over control of the amount of glucose that transferred to basolateral side from the cells

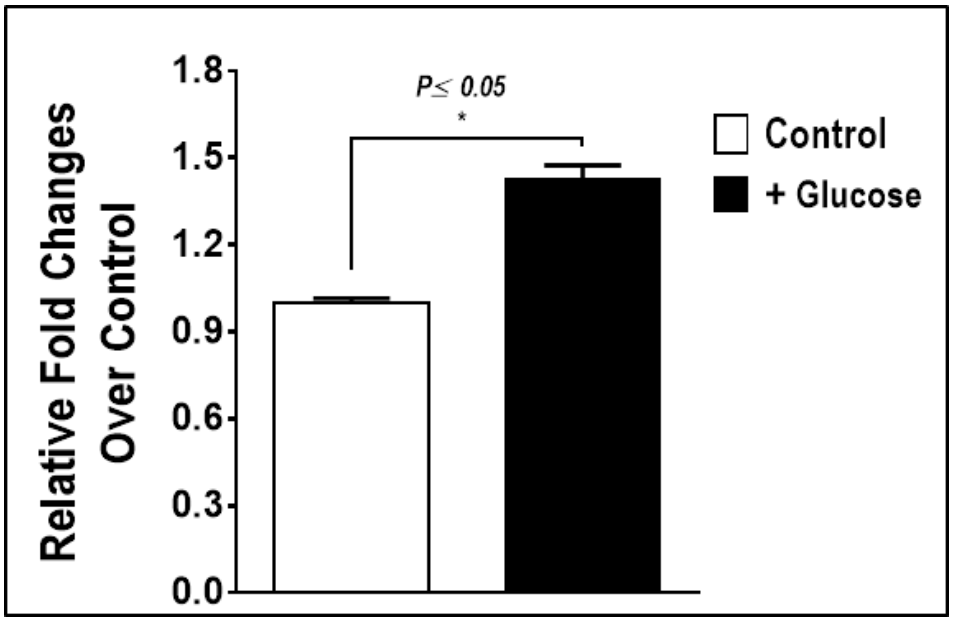

Figure 3.5. The measurement of the amount of glucose of 240th minute by Nelson- Somogyi method

3.4. Determination of Primers Quality

Primer efficiency is essential for appreciated perform of the RT-qPCR reaction. Thus, we tested primers efficiency by analyzing standard curve from cDNA samples that were diluted in different ratio. Figure 3.6 A and B show there is only one picks regarding different cDNA concentrations. Furthermore, SYBR® Green RT-qPCR protocol has melting curve steps that is used to control primer efficiency for every individual RT-PCR running and samples. The melting curve analysis for reference (GLUT2, GLUT5 and SGLT1) and housekeeping genes (CypA) were indicated on figure 3.7 and 3.8. We observed only one single pick from these gene primers indicating primers recognized only specific mRNAs of the experimental genes. Moreover, efficiency of primer amplification were analyzed for every single RT-qPCR reactions.

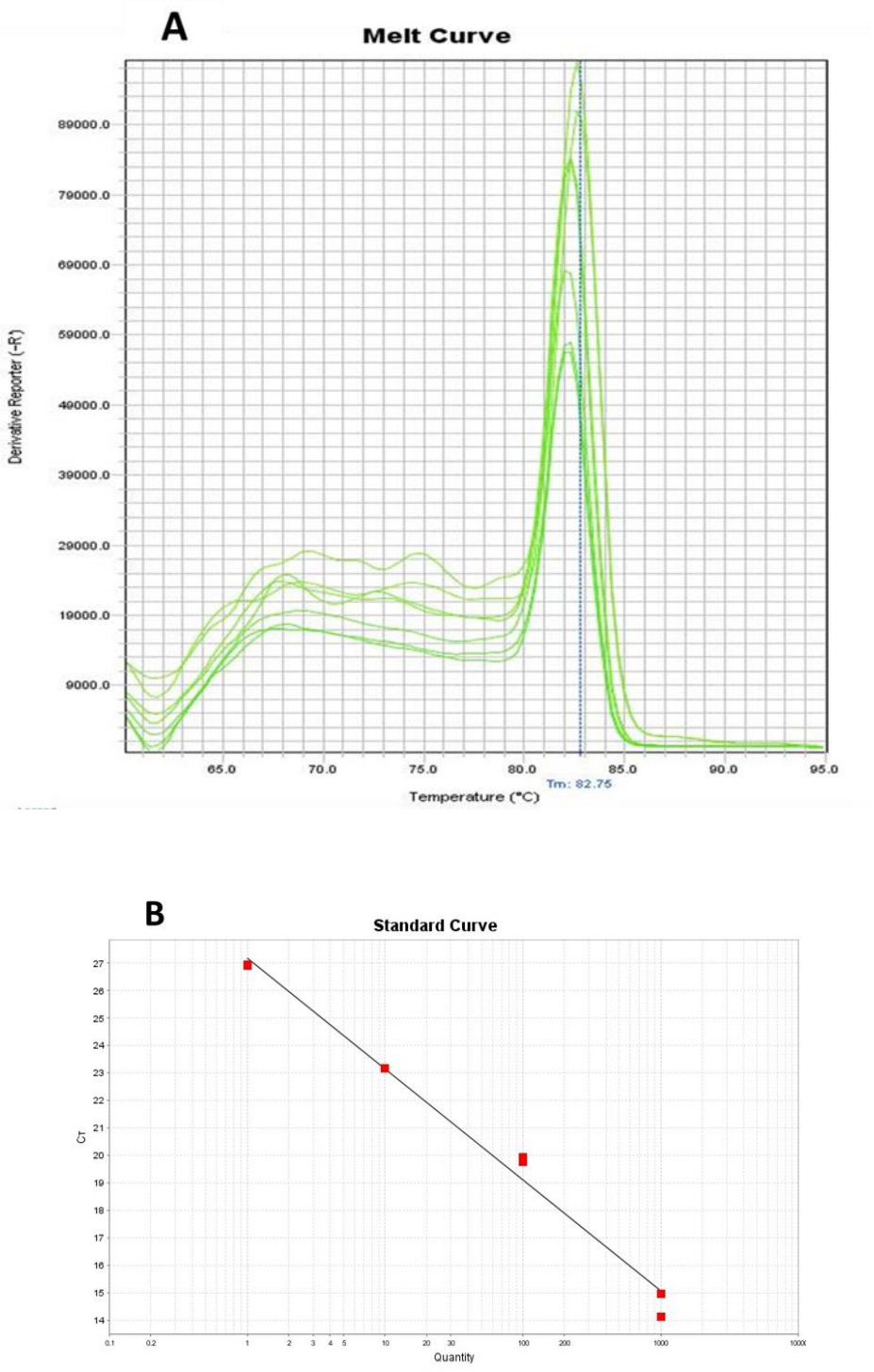

Figure 3.6. (A) shows melting curve graphic of CypA primer and (B) shows standart curve graphic of CypA primer according to standarts

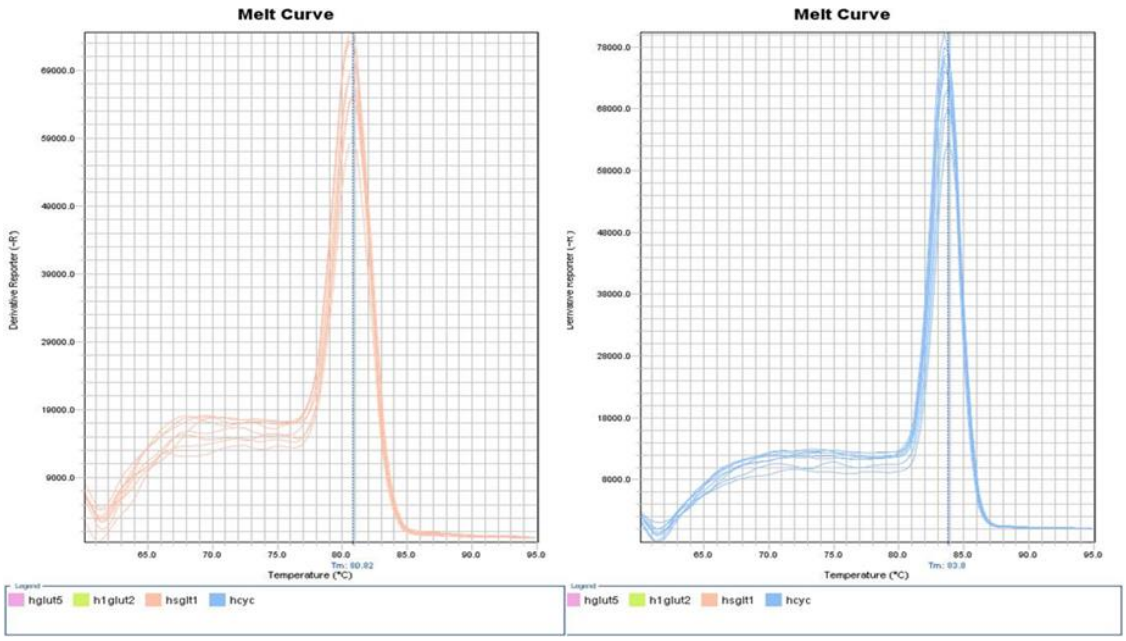

Figure 3.7. Melting curve graphics of SGLT1 primer (pink) and CypA primer (blue)

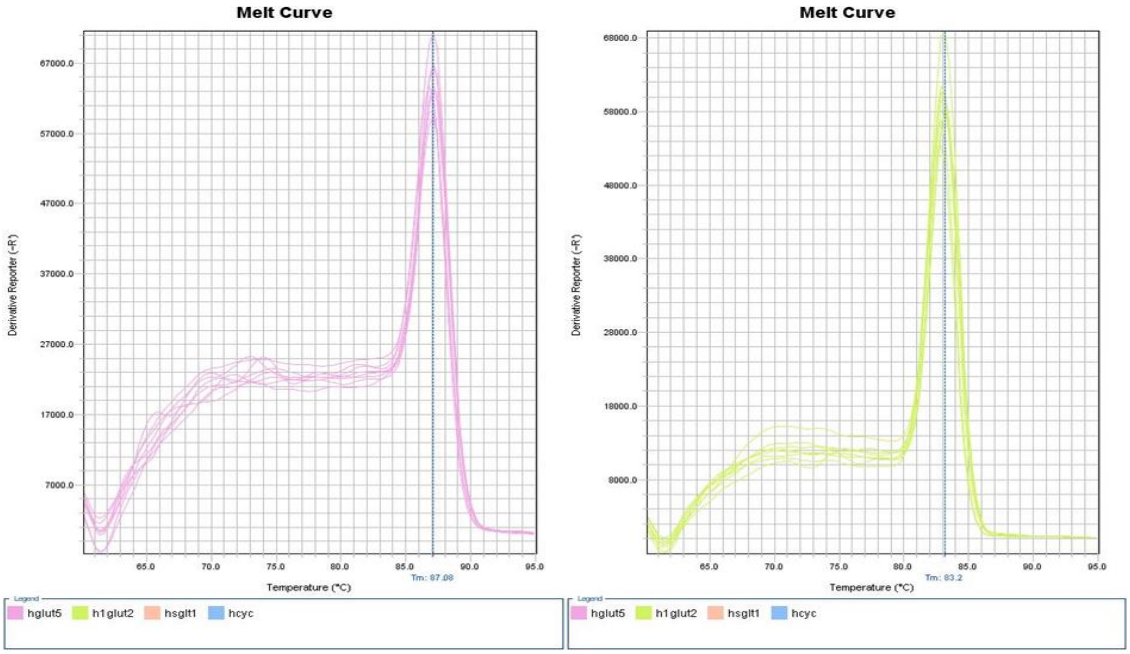

Figure 3.8. Melting curve graphics of GLUT5 primer (purple) and GLUT2 primer (green)

3.5. Evaluation of RNA Samples Quality

The quality of total RNA isolation can be controlled by looking at 18S and 28S fragments on the agarose gel. Predicted 18S and 28S fragments can be seen clearly in figure 3.9. We did not observe any degradation on bands so this indicates that of the total RNA samples have enough quality to use those samples for downstream steps including RT-qPCR and mRNA array. Moreover, the quality of RNA samples were controlled by bioanalyzer equipment before mRNA array was performed. We will discuss those result on the later part of this chapter.

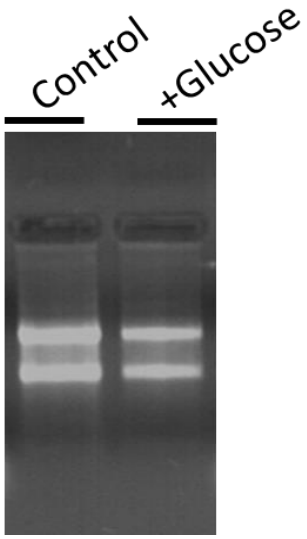

Figure 3.9. The apparition of 28S and 18S fragments on the agarose gel

3.6. Effect of Glucose Treatment on CaCo-2 Cells

It is essential to know whether our high glucose treatment affects genetic regulation of cells compared control group. To test this, we used mRNA expression levels of SGLT1, GLUT2 and GLUT5 genes as a reference maker. Firstly, the CaCo-2 cells were grown in the medium that contains 5.5mM glucose until 10^{th} day of polarization and the TEERs were measured more than 250 ohm/cm^2. The cells then were separated into two groups and one group continued to grow in 5.5mM medium and the other group started to grow in 25mM medium for 11 days more. In 21^{st} day, RNA isolation was performed following RT-qPCR. We did not observe any significant changes between groups for marker genes mRNA levels (Figure 3.10). These leaded to

change our treatment strategy. Studies showed that CaCo-2 cells might be needed to acclimation for high glucose level (Mahraoui et al., 1994). Thus, we separated cells control and high glucose groups and we plated cells at least 3 passages under 5.5mM and 25mM glucose concentration. After this step, cells were transferred on transwell system as two different groups and they were grown for 21 days. Next, RT-qPCR was performed to look at marker gene mRNA expression profile. It is observed that relative mRNA expression levels of SGLT1, GLUT2 and GLUT5 significantly decreased with high glucose treatment (Figure 3.11). This shows us our treatment strategy worked efficiently and cells were adopted high glucose condition. However, we were surprised that those mRNA expression of glucose transporters were down regulated under high glucose condition and prediction was glucose dependent induction of these genes to transport glucose from cells to basolateral site. The studies indicated that GLUT2 and SGLT1 had low Km value for glucose and they transported glucose under very low glucose concentration (Mahraoui et al., 1994). Moreover, northern blot data from same group showed that their expression levels were significantly induced with low glucose. GLUT5 is cotransporter for both glucose and fructose and it has low binding capacity for glucose than fructose and its mRNA expression levels were downregulated with high glucose (Mahraoui et al., 1994). We were not interest in SGLT1, GLUT2 and GLUT5 gene regulation in current proposal project. Main target for this experiment strategy was only to find appreciated condition that glucose has effect on molecular and genetic regulation of cells.

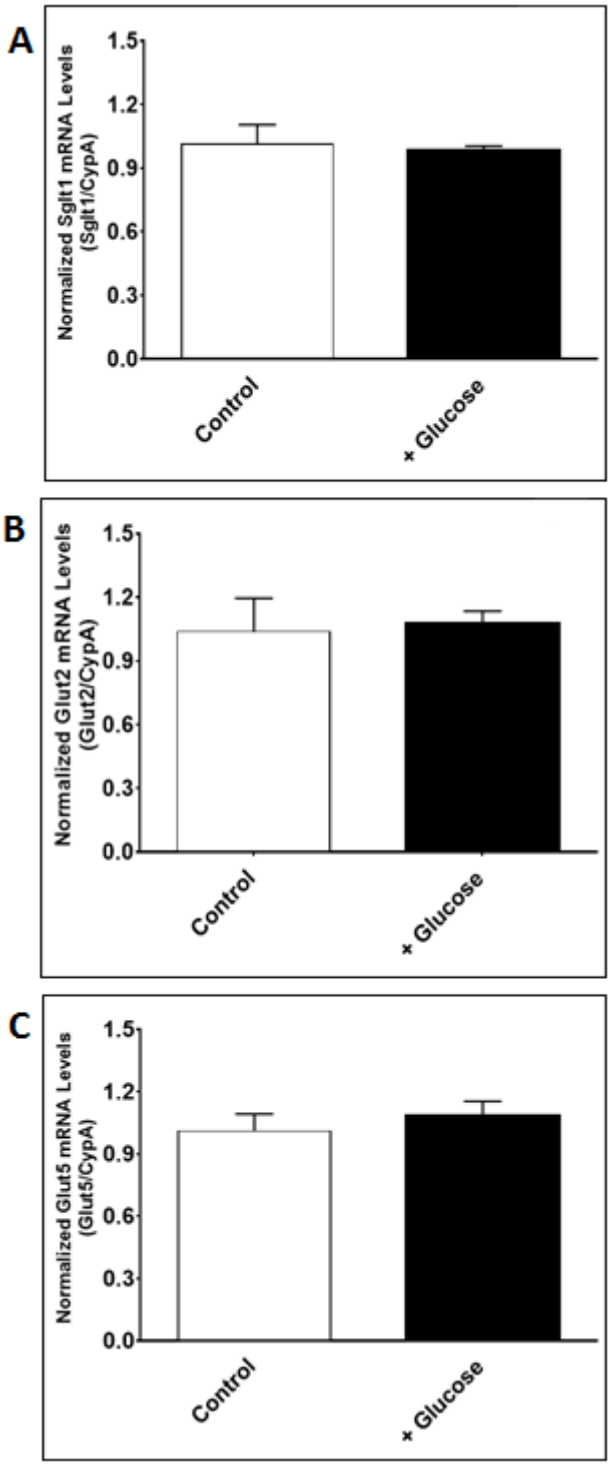

Figure 3.10. mRNA expression levels of SGLT1 (A), GLUT2 (B) and GLUT5 (C) according to first glucose treatment strategy

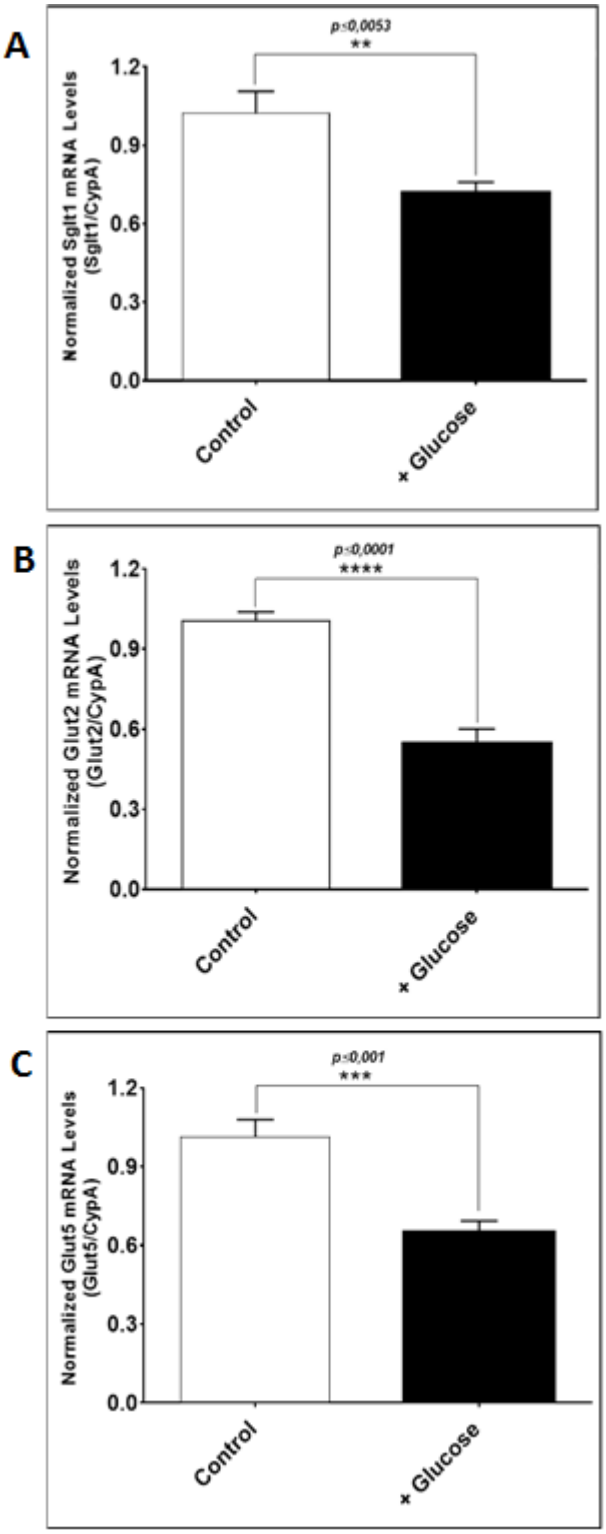

Figure 3.11. mRNA expression levels of SGLT1 (A), GLUT2 (B) and GLUT5 (C) according to second glucose treatment strategy

3.7. Genome-Wide mRNA Expression Profile

After we saw effect of high glucose on CaCo-2 cell genome, glucose dependent genome-wide mRNA expression profile was analyzed. This mRNA array technology needs to expensive equipment on hand so facilities in our university does not have this technology. Thus, this step of the project was performed in private company. However, all experimental steps were controlled closely by us. This technology contains three main steps including defining of the mRNA quality, converting RNA samples to cRNA and hybridization of samples with probes on chip, lastly analyzing data.

We isolated total RNA from experimental samples. We combined samples in separated group and run as a two samples on the agarose gel. The quality of RNA samples also were tested by bioanalyzer. According to results, 18S and 28S fragments were determined prominently on the agarose gel (Figure 3.9). This result reveals that there were not any degradation in the samples. For bioanalyzer results, 18S and 28S of samples were indicated as peaks. Pixel apparition of bioanalyzer results were determined from RNA samples that obtained from control group cells (A) and high glucose group cells (B). According to references used in bioanalyzer, 18S fragment separated from column in 43rd second and 28S fragment separated from column in 49th second. The peaks of samples can be seen clearly in figure 3.13 and their dispersement times exactly match up with dispersement times of references. After quality quantification, samples were converted to cRNA hybridization with 47.000 probes containing chip (HumanHT-12 v4 Expression BeadChip) and output signals were read to see crude signals from probes.

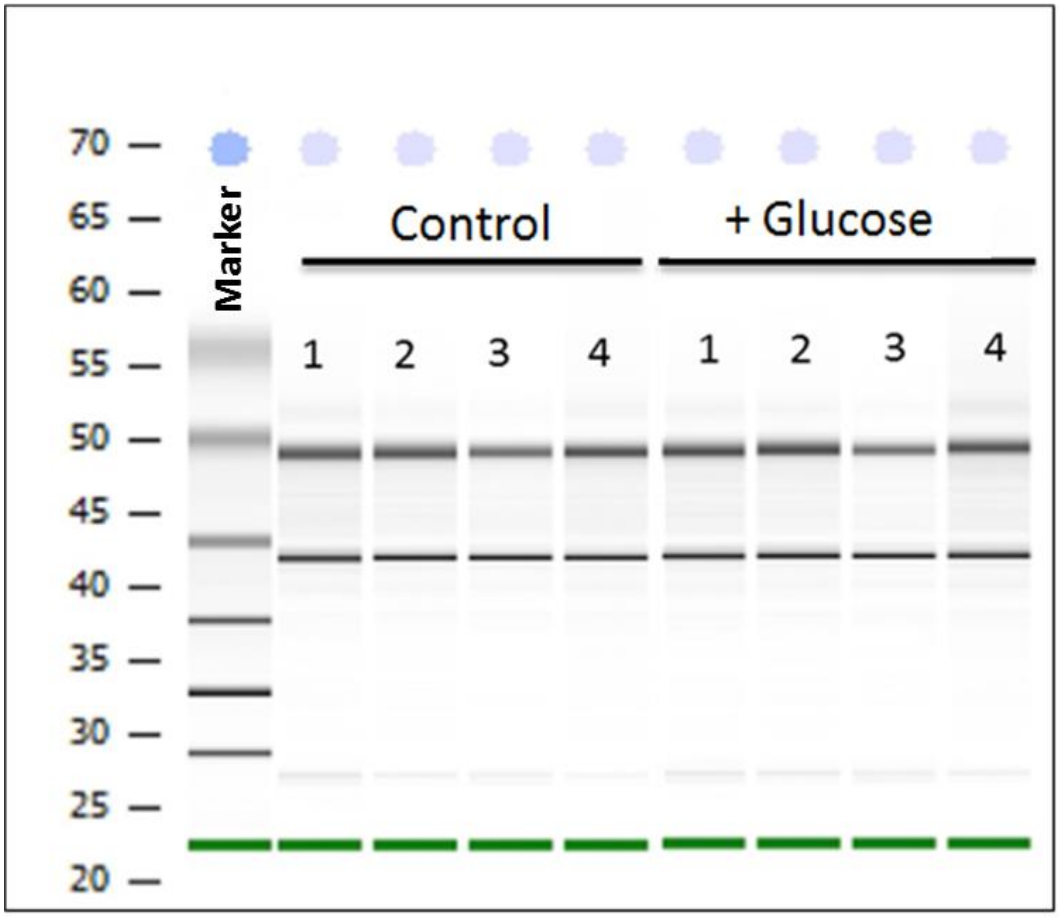

Figure 3.12. Bioanalyzer results of RNA samples

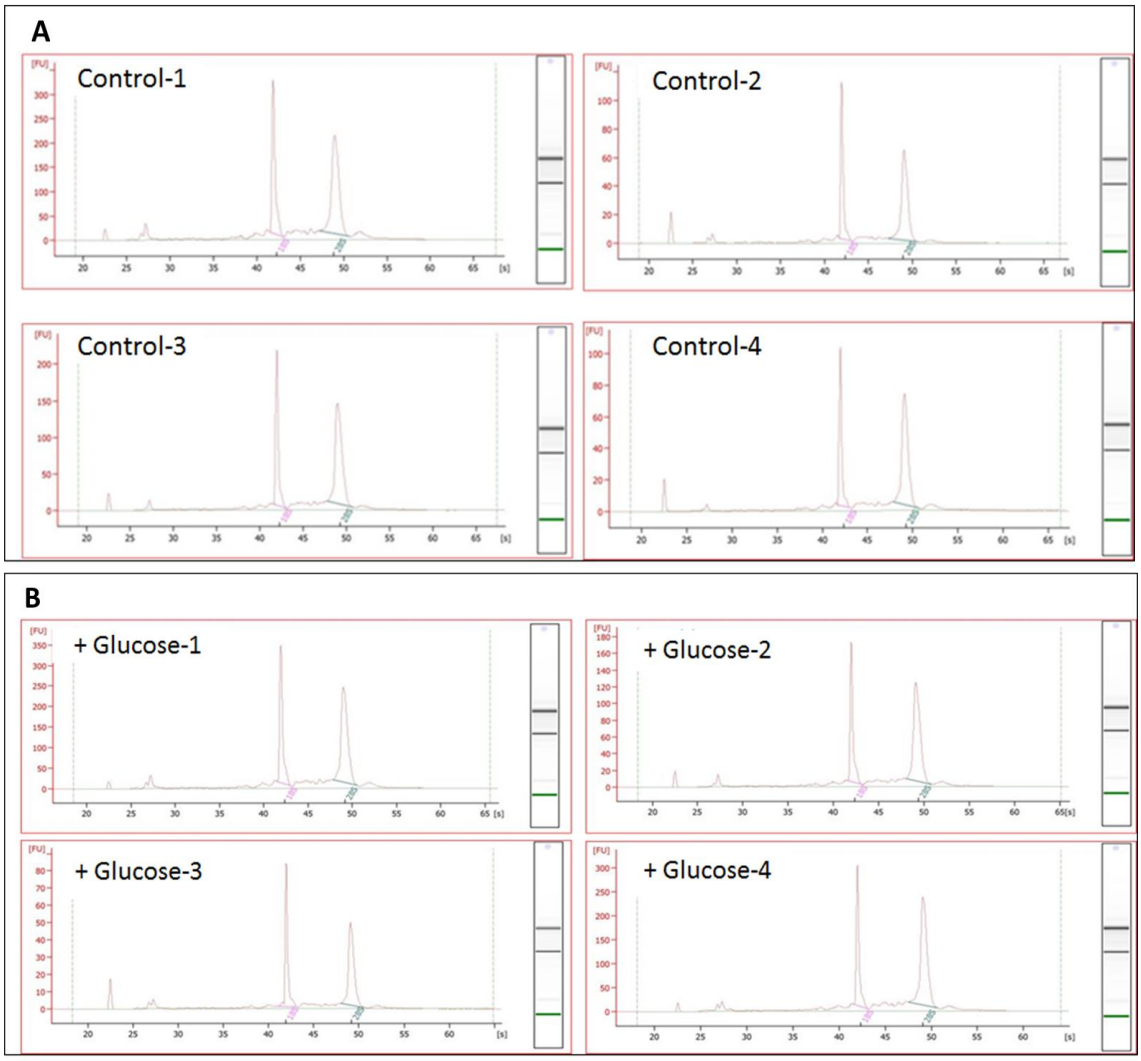

Figure 3.13. Pixel apparition of bioanalyzer results of RNA samples for control groups (A) and high glucose groups (B)

3.8. Data Analysis

Crude microarray data was converted to electronic data by GenomeStudio program. Signal intensity graphics due to control purposes created of microarray data are shown in the following graphics. The quantiles of all samples data was subjected to normalization. The scatterplot is shown in figure 3.14 and 3.15.

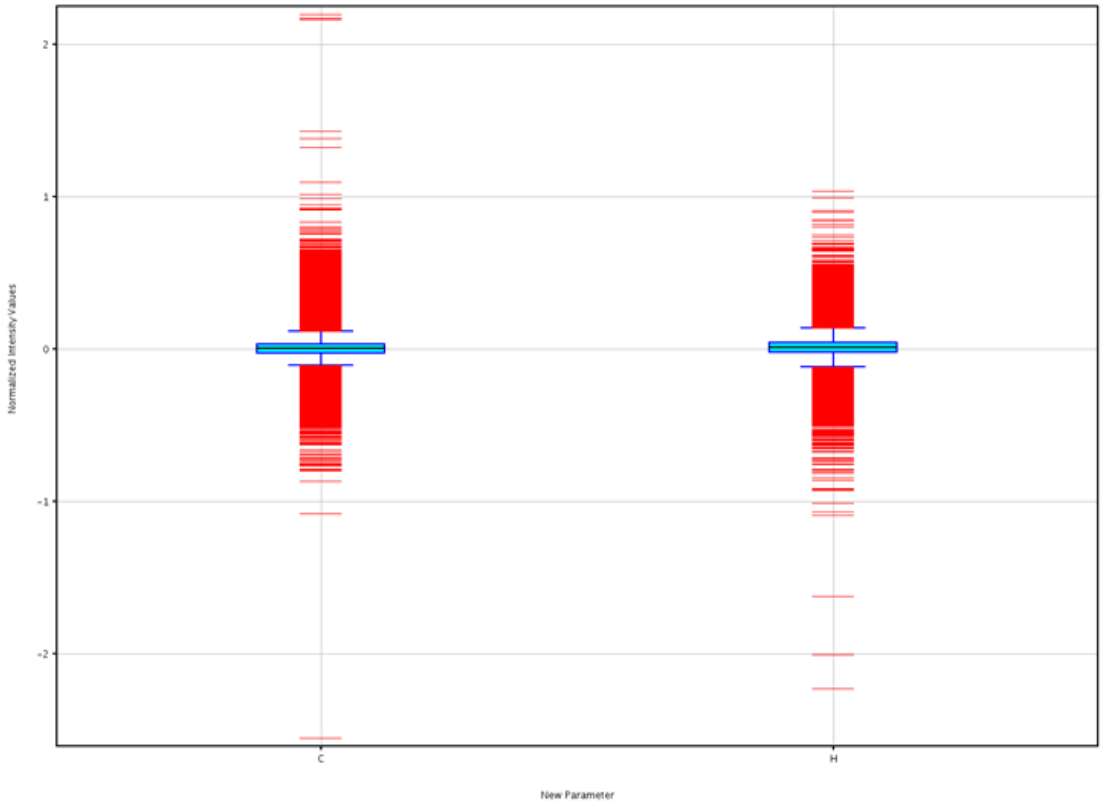

Figure 3.14. Profile Plot of all the samples

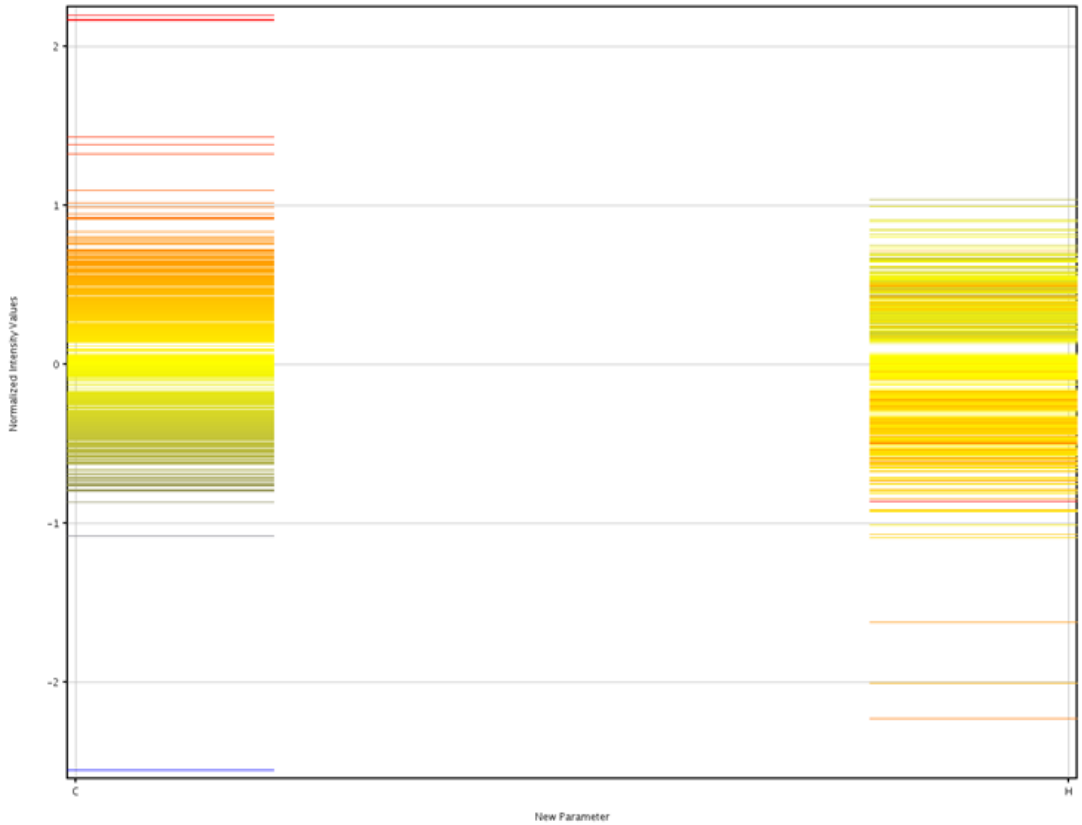

Figure 3.15. Box Plot of all the samples

Cluster analysis was performed after all the gene expression profiles were normalized. Cluster analysis was formed by hierarchical approach. The distance unit was chosen as Euclidean and the connection principle was chosen as Wards. Cluster analysis of groups data obtained from calculated data of probe luminescence are shown in figure 3.16 and 3.17. The red color indicates down regulation, the green one is up regulation and the black one shows that there are limited changes in the level of genes. Figure 3.16 also infers the proximity of around 47.000 probes so accordingly, the proximity of genes.

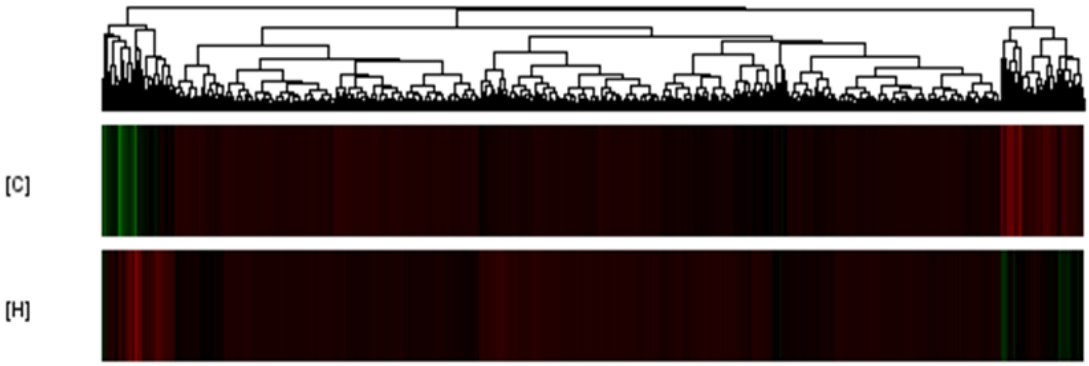

Figure 3.16. Cluster analysis according to samples

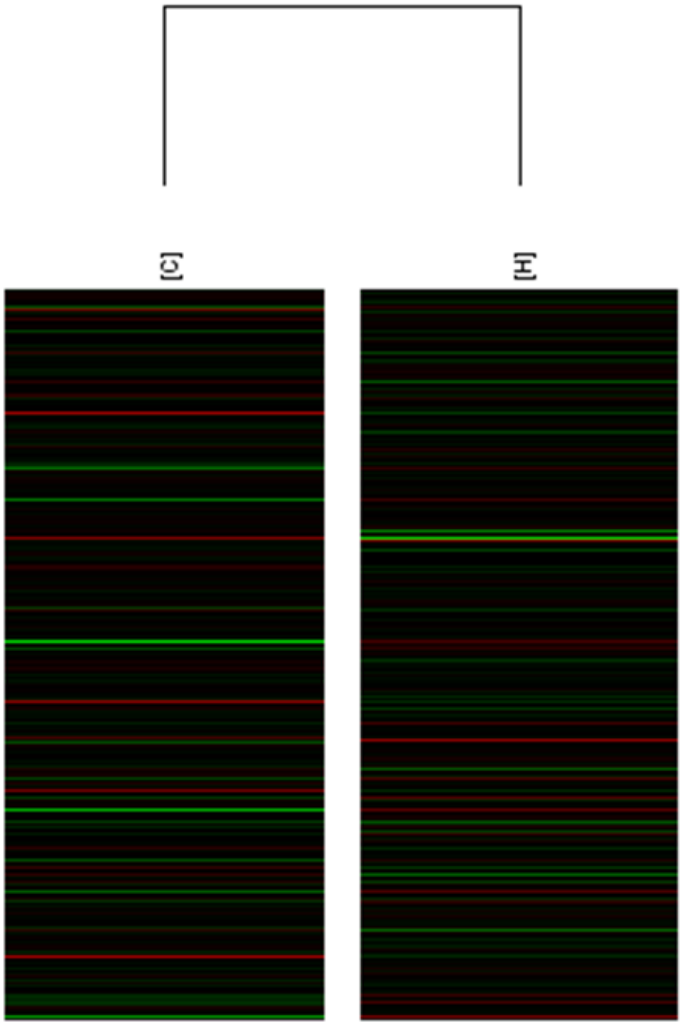

Figure 3.17. Cluster analysis according to groups

Assay started with 47.000 probes. These probes were filtrated according to their expression levels. The probes that we received luminescence remaining under 20% of normalized data were tripped over the filter. If over 20% luminescence was received from a probe in any group, it was allowed to pass through the filter and thus, probe loss was minimized. After, fold change (FC) analysis was performed and more than 1.5 fold changes were stated for each probes as up or down regulated. Heat-maps for up and down regulated probes are shown in the figure 3.18 and 3.29. Upregulated probes are indicated as green and downregulated probes are indicated as red in the heat-map. The black sides demonstrate that there is not significant changing in the gene. According to FC analysis from filtered samples that expression of 351 probes were increased and expression of 468 probes were decreased compared to the control group.

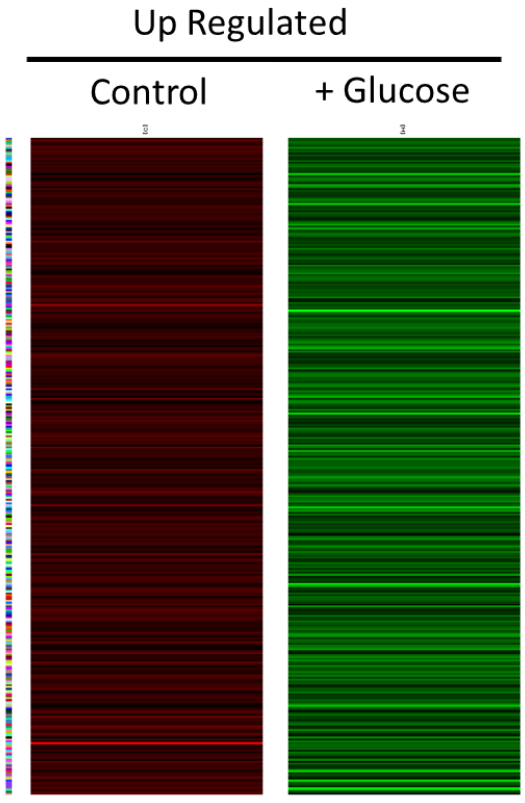

Figure 3.18. Heat-map for upregulated probes of high glucose group vs control group

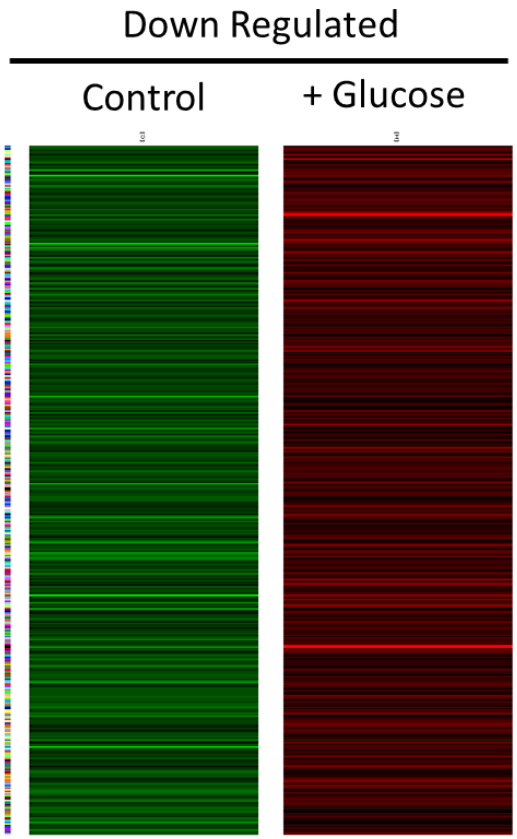

Figure 3.19. Heat-map for downregulated probes of high glucose group vs control group

3.9. Regulated Genes and Metabolic Pathways

DAVID bioinformatics online analysis open source program was used to find affected pathways. Normalized row data was analyzed for significance between control and high glucose treated groups for down and up regulation. We selected mRNAs at the level of 1.5 fold or more changes between groups to perform pathways. At the first step, significantly affected genes expression profile were determined and then these genes were clustered to find which genes play a role in which physiological pathways. When down regulated genes were clustered, glycolysis, pentose phosphate, fructose and mannose, insulin signaling, inositol, adherens junctions pathways were affected. As contrast, protein export and spliceosome mechanism were affected regard to upregulated gene cluster analysis. However, *DAVID* pathway analysis indicated that glycolysis, pentose phosphate, fructose and mannose, adherens junction pathways were significantly downregulated whereas protein export pathway was significantly upregulated. Thus, we looked at individual genes that might play a role in specific

pathway which did not show up on *DAVID* analysis program. We found significantly downregulated genes that play role on insulin and inositol pathways. Moreover, we observed that few genes with unknown function in intestine were shown regulated between groups. In this and next parts we will only focused on metabolic pathways and genes that are closely related to obesity and diabetes in the literature.

Energy requirements in body from glucose are provide from glycolysis or other energy yielding pathways. When the cells do not need energy those molecular mechanisms should be reduced. In our data shows that glycolysis, pentose phosphate and fructose, mannose pathway were significantly downregulated consistence to physiologic response of cells during accessible high glucose condition. High glucose was significantly reduced to adherens junction pathway in our minimalistic *in vitro* intestine systems. Junctional interaction between enterocytes has a vital role for intestinal barrier function and it is also important for regulation of intracellular molecular pathways. This cellular changes in our experimental model how impact to glucose metabolism on enterocytes is needed further investigations. When we compared pathways regulation in terms of the high glucose, we observed that numbers of the significantly downregulated molecular pathways were higher than upregulated ones. Pathways analysis indicated that intracellular protein export mechanism was significantly upregulated, during high glucose treatment. The cells use proteins including transporters, enzymes and hormones to control physiological changes regarding different environmental conditions. Their activity can be regulated posttranslational modifications by moving proteins from one compartment to another compartment of the cells. We thought that upregulation of the intracellular protein export pathways was general response of the cell to high glucose and this might not be directly involved in obesity and diabetes. The data from this project provided to us new specific genes that can be subjected new future projects.

3.10. Pathways Significantly Downregulated in High Glucose Condition

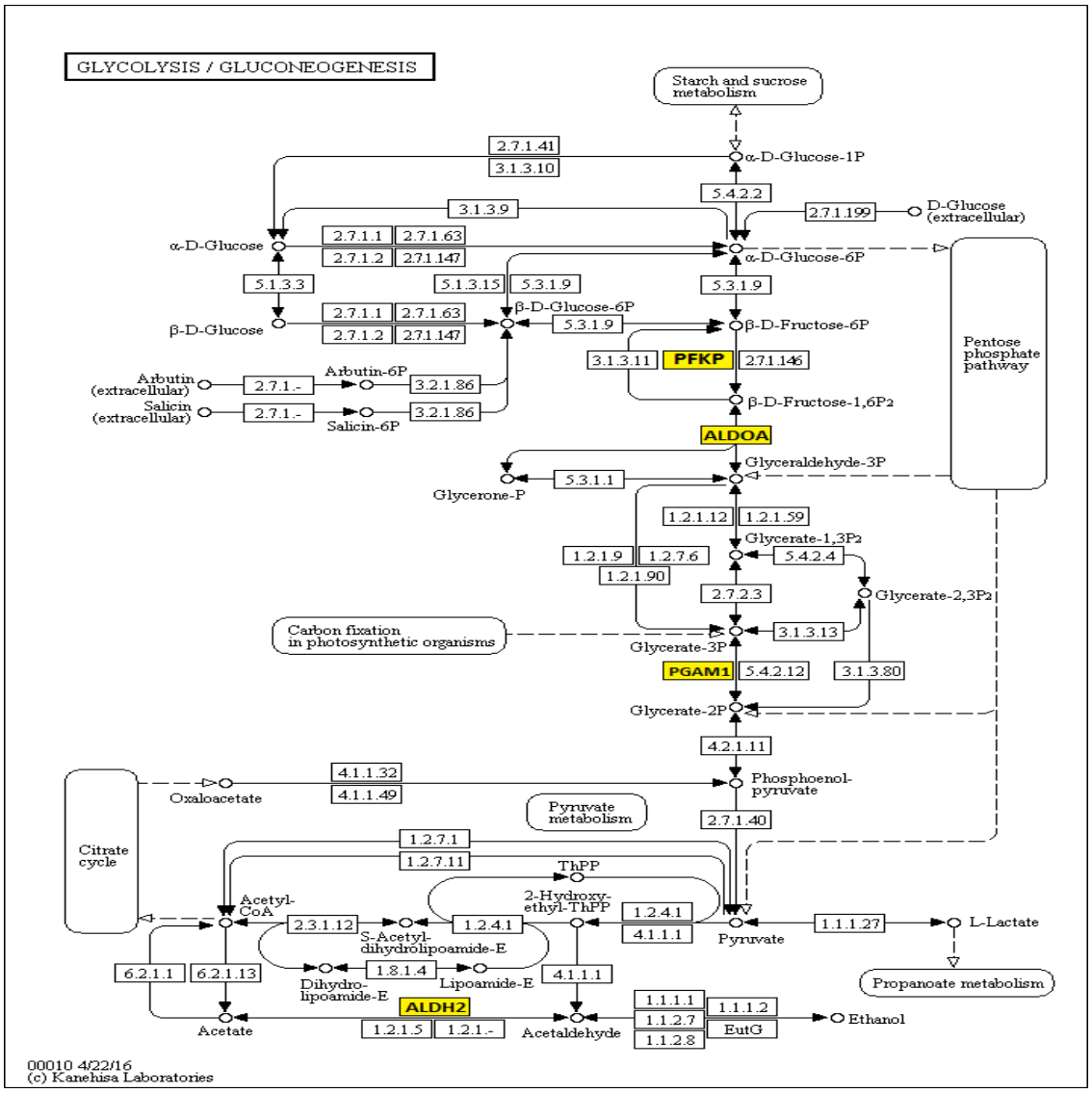

Figure 3.20. Glycolysis/Gluconeogenesis Pathway (P-value: 0.00003)

Table 3.1. Significantly downregulated genes of glycolysis/gluconeogenesis

Gene	P	Fold Changes
PFKP phosphofructokinase, platelet	0,0004	1,95 Down
ALDOA aldolase A, fructose-bisphosphate	0.04	2,8 Down
PGAM1 phosphoglycerate mutase 1 (brain)	0.04	2,2 Down
ALDH2 aldehyde dehydrogenase 2 family (mitochondrial)	0.003	1.59 Down

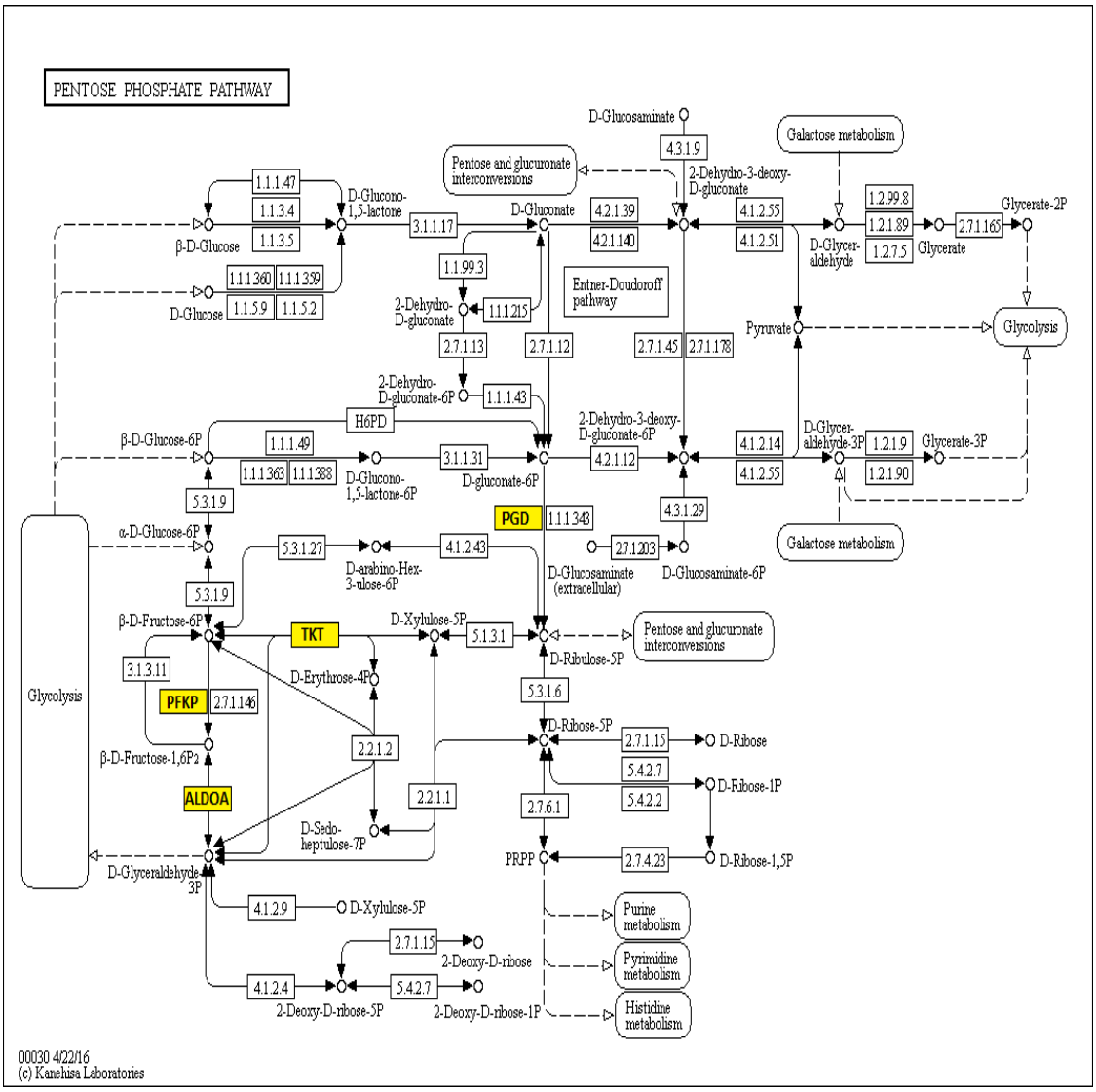

Figure 3.21. Pentose Phosphate Pathway (P-value: 0.0043)

Table 3.2. Significantly downregulated genes of pentose phosphate pathway

Gene	**P**	**Fold Changes**
PGD phosphogluconate dehydrogenase	0.01	1,6 Down
TKT transketolase 1	0.006	1,6 Down
PFKP phosphofructokinase, platelet	0, 0004	1,95 Down
ALDOA aldolase A, fructose-bisphosphate	0.04	2,8 Down

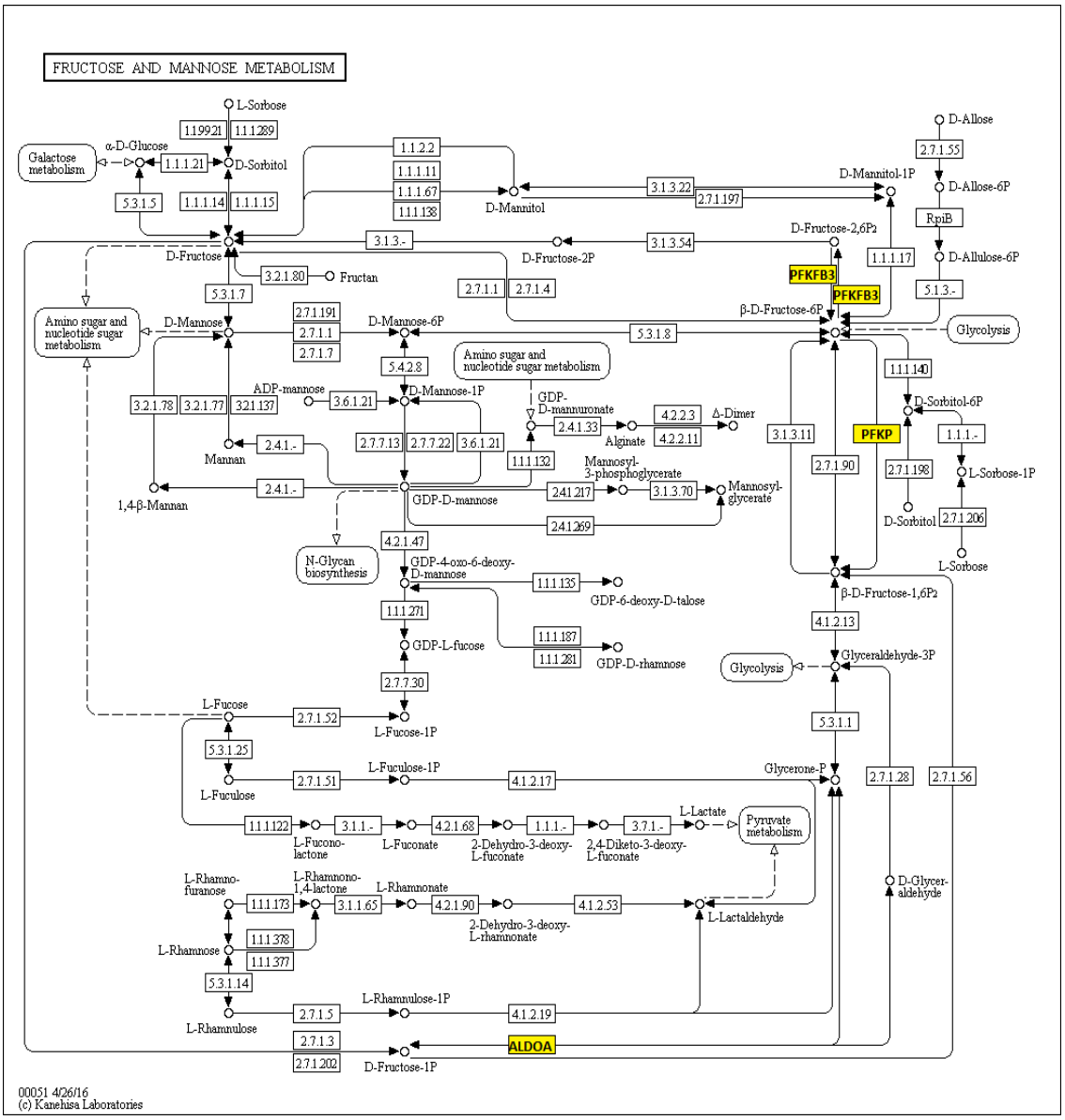

Figure 3.22. Fructose and Mannose Metabolism Pathway (P-value: 0.013)

Table 3.3. Significantly downregulated genes of fructose and mannose metabolism

Gene	**Gene**	**Gene**
PFKFB3 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	0.001	1,8 Down
PFKP phosphofructokinase, platelet	0,0004	1,95 Down
ALDOA aldolase A, fructose-bisphosphate	0.04	2,8 Down

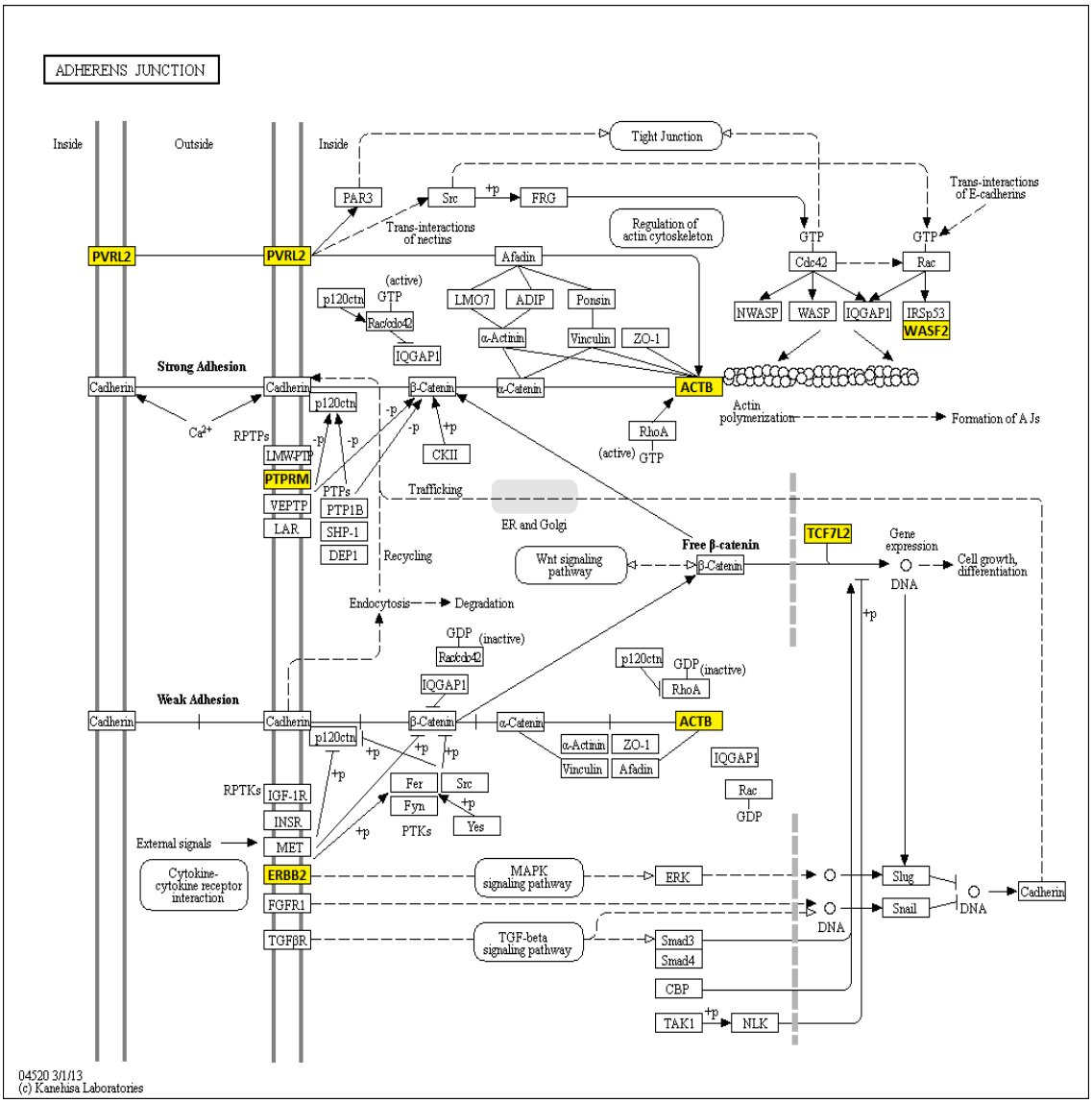

Figure 3.23. Adherens Junction Pathway (P-value: 0.0011)

Table 3.4. Significantly downregulated genes of adherens junction

Gene	**P**	**Fold Changes**
PVRL2 poliovirus receptor-related 2	0.03	1,5 Down
ACTB actin, beta	0,002	3,1 Down
WASF2 WAS protein family, member 2	0.001	1,7 Down
PTPRM protein tyrosine phosphatase, receptor type, M	0.004	1,9 Down
TCF7L2 transcription factor 7-like 2 (T-cell specific, HMG-box)	0,0004	1,6 Down
ERBB2 v-erb-b2 erythroblastic leukemia viral oncogene homolog 2	0.005	1,7 Down

3.11. Pathways Significantly Upregulated in High Glucose Condition

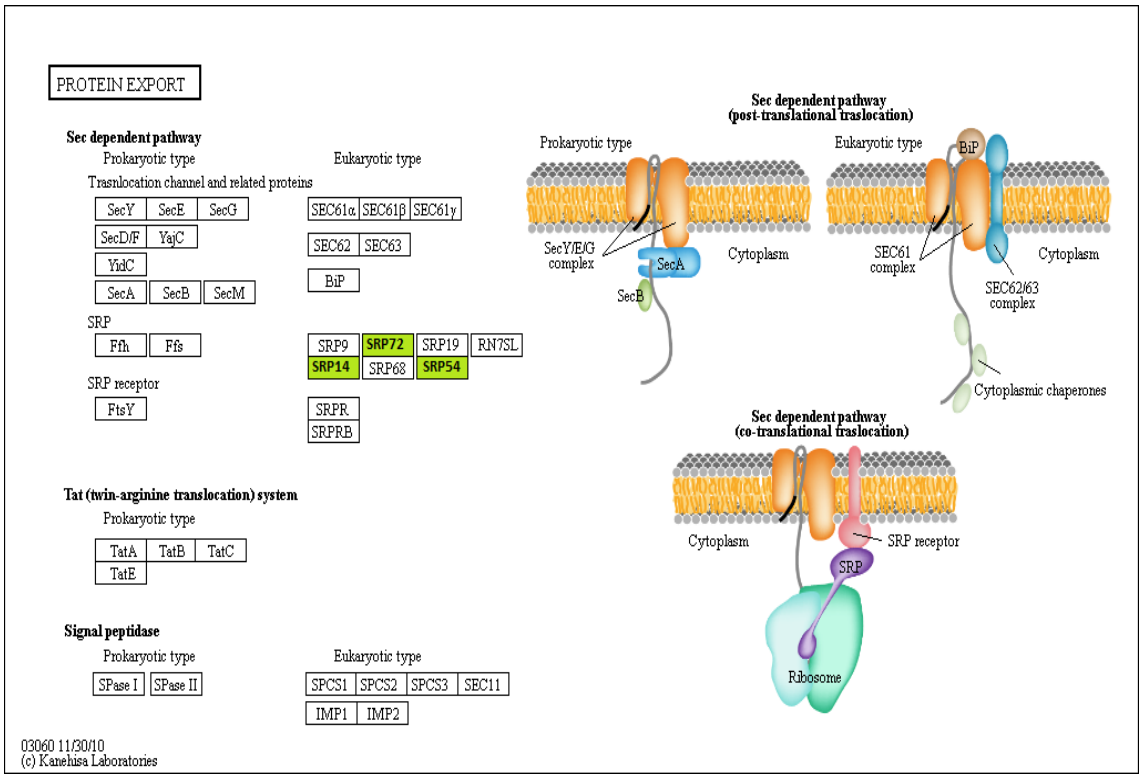

Figure 3.24. Protein Export Pathway (P-value: 0.0069)

Table 3.5. Significantly upregulated genes of protein export

Gene	P	Fold Changes
SRP14 signal recognition particle 14kDa	0,00017	1,6 Up
SRP72 signal recognition particle 72kDa	0,02	1,5 Up
SRP54 similar to signal recognition particle	0.00035	1,6 Up

3.12. Confirmation of mRNA Levels by RT-qPCR

In this proposal we tended to investigate find effect molecular pathways that might play a role in intracellular glucose metabolisms by mimicking *in vivo* intestine system of obese people. Because, intestine of obese people are exposed high glucose containing food and consequently high blood glucose. We looked at genes mRNA levels that have role on glycolysis, pentose phosphate, and fructose and mannose pathways. This is also important to confirm our mRNA array results. We found that mRNA levels of the ALDOA, ALDH2, PFKP, PGAM1, PGD and PFKFB3 were significantly down regulated in high glucose treated cells and this data are correlated with mRNA array data (Figure 3.25). ALDOA, ALDH2 and PGAM1 are involved in glycolysis which is cellular production of the energy (Frohnert et al., 2011), (Fernandez-Trasancos et al., 2014). In high glucose condition, the cells do not need further metabolize glucose for energy requirements. PFKP plays a role in glycolysis, pentose phosphate and fructose/mannose pathways and the studies showed that bases changes on this gene were associated with increased obesity and T2D risks (Scuteri et al., 2007), (Morgan et al., 2010). It was found that PGD and PFKFB3 proteins involved in pentose phosphate and fructose/mannose pathways respectively. According to the studies of Akbay et al. (2004), the mRNA level of PGD reduced in liver of rats with high fat diet which shown moderate obesity, insulin resistance, hyperglycemia and hyperlipidemia. PFKFPB3 mRNA levels also were downregulated during high level adiposity and obesity (Jiao et al., 2008). Our results were similar to literature for regulation of these genes in obesity and T2D. Thus, the regulation of these genes in model of the fully differentiated human intestine system suggest that enterocytes cell might be involved in glucose induced obesity and T2D. However, these observations should be evaluated in animal models at the levels of protein and function.

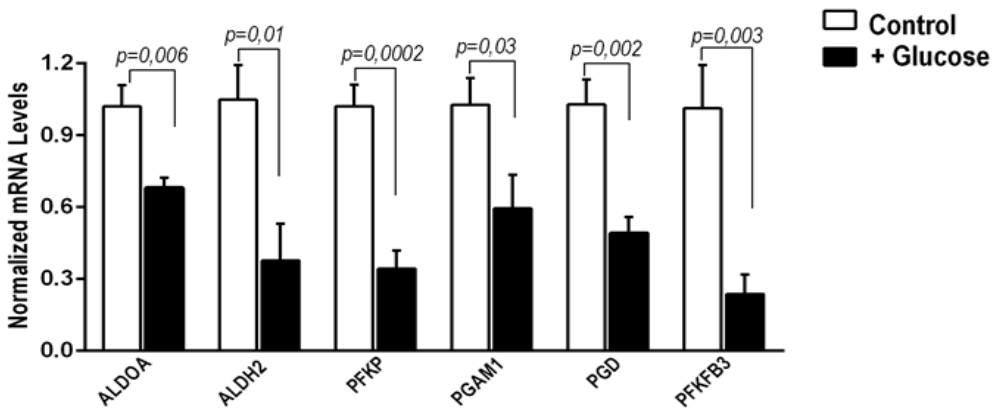

Figure 3.25. Confirmation of the mRNA Levels of ALDOA, ALDH2, PFKP, PGAM1, PGD and PFKFB3 by RT-qPCR

Some of the genes from array data were significantly down regulated and they have role in insulin pathway. Pathway analysis data surprisingly showed that insulin signaling and inositol phosphate pathways did not reach significance ($p<7.5x10^{-2}$, $p<5.9x10^{-2}$). However, when we looked at gene mRNA expression levels, we found that the genes for insulin signaling and inositol phosphate pathways were significantly downregulated under high glucose condition (Figure 3.26).

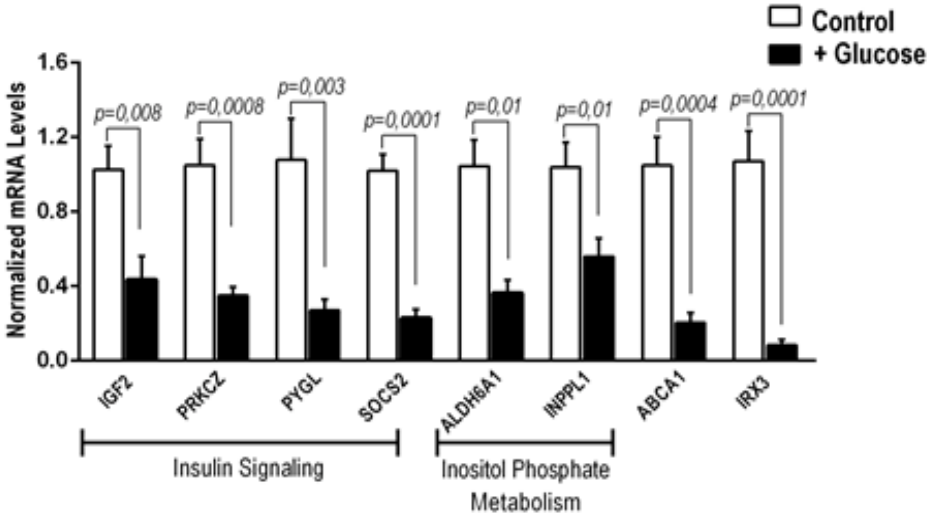

Figure 3.26. Confirmation of the mRNA Levels of IGF2, PRKCZ, PYGL, ALDH6A1, INPPL1, ABCA1 and IRX3 by RT-qPCR

Table 3.6. The abbreviations of the genes from figure 3.26

ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1
ALDH6A1	Aldehyde dehydrogenase 6 family, member A1
IGF2	Insulin-like growth factor 2
INPPL1	Inositol polyphosphate phosphatase-like 1
IRX3	Iroquois homeobox 3
PRKCZ	Protein kinase C, zeta
PYGL	Phosphorylase, glycogen, liver

It has been known that pancreas tissue secretes insulin and regulates mainly GLUT4 dependent glucose uptake in muscle and adipose tissues (Leto & Saltiel, 2012). It was indicated that the insulin signaling pathway dependent genes from figure 3.26 were involved in obesity and T2D. For instance, IGF2 is a poly peptide hormone and its overexpression leads excessive growth of the adipose tissues (Kadakia & Josefson, 2016). IGF2 plays a role on glycogen production through Akt pathway in liver. In our study glucose significantly (2 fold, $p<0.01$) reduced to IGF2 mRNA levels. This might be important for physiological balance of glucose and production of the glycogen in our experimental model. Because, human intestine is second tissue after liver to provide energy for body during low energy state. When we analyzed the mRNA data, we found that ALDH6A1 and INPPL1 genes that play role in inositol phosphate metabolism and were associated with T2D. It has been found that ALDH6A1 in adipose tissue was downregulated during obesity and T2D (Dharuri et al., 2014) and INPPL1 knockout mice highly resistant to weight gain under high fat diet (Sleeman et al., 2005). In our study we found that high glucose significantly reduced ALDH6A1 and INPPL1. This reduction might be related to protective or adaptive mechanism to high glucose in enterocyte of intestine.

Other than the genes that play a role in above indicated metabolic pathways, we were interested in looking mRNA expression levels of ABCA1 and IRX3. These genes are involved in cholesterol and fat metabolism. The studies suggest that their role in adiposity might regulate secretion of adipokines from adipose tissue (de Haan, Bhattacharjee, Ruddle, Kang, & Hayden, 2014), (Ronkainen et al., 2015). Moreover, uridine rich small RNA family members, RNU1-5 (8 Fold, $p<0,001$), RNUG2 (7,6 Fold, $p<0,001$), RNU1-3 (7,5 Fold, $p<0,00$), were significantly down regulated as

cluster of genes in our array data. They are expressed ubiquitously in eukaryotic cells, but we do not know how this gene cluster play a role into enterocyte cells during high glucose condition. Until now, we focused on down regulated genes and their functions in different tissues during obesity and T2D. Our results from enterocyte cells are correlated to regulation of the genes with literature. This suggests that enterocyte cells might behave other tissue including adipose, pancreases, and muscle in terms of the obesity and T2D. However, enterocyte specific regulation in obesity or diabetes should be investigated by looking of the genes functions. This will provide more mechanistic studies about role of the enterocyte cells in metabolic diseases.

Interestingly, numbers of the genes, which were significantly changed between groups, were observed in down regulated group (at least 2.0 fold or more 72 genes) than upregulated group (at least 2.0 fold or more 31 genes). When we performed *DAVID* analysis for upregulated genes, only intracellular protein export pathways showed up regard to high glucose and we could not find any physiologic interaction of this pathway to obesity and diabetes progression in the literature. Thus we focused on individual genes to confirm mRNA levels by RT-qPCR. For instance, high glucose induced significantly (7,6 fold, p=0.004) mRNA level of TXNIP gene and also we observed significant induction (3.5 fold, p=0.0001) of this gene by RT-qPCR (Figure 3.27). We observed level of the fold differences between from array and RT-qPCR data. This might be due to different principle of these two technologies. TXNIP includes in varies metabolic diseases (Gondo, Satsu, Ishimoto, Iwamoto, & Shimizu, 2012). It has been reported that TXNIP plays a role in glucose uptake (Yu, Goh, Dai, & Luo, 2009) and glucose production in liver of mice (S. T. Hui et al., 2008). TXNIP mutant mice showed hypertriglyceridemic and hypoglycemic during fasting state (T. Y. Hui et al., 2004). These observations suggest that TXNIP might be important regulator candidate for glucose metabolism and obesity in intestine. We found that another gene, lipocalin 15 (LCN15) was significantly induced in high glucose treated cells (Figure 3.27). There is very limited information about LCN15 in the literature. However, LCN2 which is another family member of LCN, is best studied gene in the literature. LCN2 is called adipokine and it is secreted from adipose tissue. LCN2 expression is induced in obese human and different obese and T2D mouse models (Guo et al., 2010). LCN2 null male mice shows protecting from high fat diet induced obesity and insulin resistance (Law et al., 2010). It was concluded that LCN2 might be regulator in adipose tissue to modulate glucose metabolism in murine tissues and in 3T3-L1 adipocytes (Yan et al., 2007). In

our experimental models, RT-qPCR results showed that LCN15 was increased around 6 fold by high glucose and this bring the idea that LCN15 might be secreted from enterocytes in terms of the high glucose condition to stimulate different cell types of intestine peripheral tissues. We definitely need more definitive data to show both TXNIP and LCN15 functions in enterocytes during obesity.

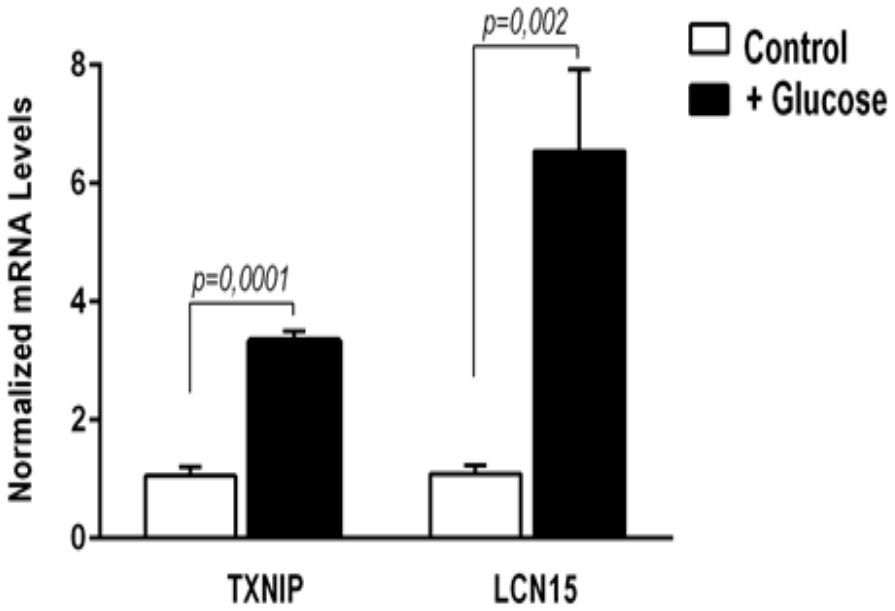

Figure 3.27. Confirmation of the mRNA Levels of TXNIP and LCN15 by RT- qPCR

Table 3.7. The abbreviations of the genes from figure 3.27

LCN15	Lipocalin 15
TXNIP	Thioredoxin interacting protein

This is first study to show enterocytes specific gene regulation during chronic high glucose treatment of fully differentiated human (CaCo-2) intestinal epithelial cell culture model. More proteomic and functional studies are needed to make more clear and physiologic conclusions from our data. However, this project provided valuable information for further investigations about intestine and its role in obesity.

CHAPTER 4

CONCLUSION

Obesity and type 2 diabetes (T2D) are burden diseases in the world and their prevalence gradually increases all over the world. Education programs and awareness of these diseases are established especially for younger ages. Moreover, people from all ages are coordinated to lose weight programs against to development of obesity and T2D. However, real question is “How are we successful to decrease rate of these metabolic diseases in populations?” There are great impacts of the factors that are related to our health including decreased mobility, less physical activities, and uncontrolled dietary regimes. One important behavior with living organism have is consuming of the foods. This is only way to survive in their environment. Types of the food on the market have direct effect on our health. The most food companies provide different foods without thinking health effect instead they concern cheap fabrication of the foods and longer time stock stability. These products on the market are consumed by all age groups because they are cheap, easy accessible, and very common around of us. Carbohydrates are main substances of natural and fabricated foods. When we think dietary carbohydrates, at least half of the disaccharides are consisted of glucose. Glucose is mainly used added sugar in food products to give sweet taste. Our daily diets have different level of glucose containing of food and its consumption is increased time to time. Glucose is main contributor of obesity and T2D. After glucose is consumed, it is absorbed through enterocyte cells of the intestine and into body. In human body glucose is first taken up in adipose and muscle tissues by insulin-glucose transporter 4 (GLUT4) dependent manners. If any functional problems are seen between insulin and GLUT4 interaction, either type 1 (insulin absent) or type 2 (insulin resistance) diabetes occurs. Glucose is used first energy source of the brain cells and in excess it can be converted to fatty acid and stored as fat in adipose tissues. This causes increased body weight and later on obesity. Most studies have investigated adipose, liver, hypothalamus, and pancreas tissues in terms of the development of obesity and diabetes (Bloomgarden, 2008). However, new interest is raised on intestine tissue to investigate relation between nutrients and obesity progression. Enterocytes are found with 90% ratio among of other types cells of intestine and they are regenerated every 3-4 days

(Vereecke et al., 2011). Enterocytes provide 20-25% of energy needs for human body during fasting states (Mithieux & Gautier-Stein, 2014). Furthermore, receptor of the leptin was found enterocytes and mice lacking with leptin receptor in only intestine leads to increase development of onset obesity (Montague et al., 1997). Dietary nutrients first interact with enterocytes cells and this relation requires nutrients absorption into body. Intestine system including enterocytes have mechanisms either define control nutrients types and nutrients concentrations from diets. Enterocyte cells also are regulated by various chemosensory signals including nutrients, non-absorbable food products, microbial flora and their metabolites (Shirazi-Beechey, Moran, Batchelor, Daly, & Al-Rammahi, 2011). These indicate that intestine is biologically very active tissue and directly involved in physiologic response of the body. In current project we aimed to identify molecular and genetic responses of enterocytes cells under high glucose conditions. Obese people mainly choose big size portion of food and eat food more frequently. They also absorb more dietary sugars from foods. Consequently, they have high blood glucose levels. These indicate that enterocytes of obese people are exposed high level of dietary or blood glucose molecules. Human intestine system was modeled as *in vitro* by growing CaCo-2 cells on transwell cell culture plates to mimic *in vivo* intestine system. The cells were treated 25mM glucose containing medium to create dietary and blood high glucose conditions. After genome-wide array performed, *DAVID* pathway analysis indicated that glycolysis, pentose phosphate, fructose and mannose, adherens junction pathways were significantly downregulated whereas intracellular protein export pathway was significantly upregulated. Moreover, we found that specific genes did not showed on pathways analysis results, but their expression levels were significantly regulated by high glucose and they have role in glucose metabolism, obesity, and T2D in the literature.

Experimental data in this proposal indicates that high glucose is changed some molecular and genetic response of fully differentiated human (CaCo-2) intestinal epithelial cell model. The screening of the genetic regulation in enterocyte cells provided significance target genes to investigate role of the enterocytes in obesity and T2D. As a future direction that we will focus on; *i- How is glucose regulated genes involved in enterocyte carbohydrate metabolism as protein and functional levels? ii- Is there any enterocytes specific molecule(s) that regulate other cell types on intestine system including endocrine, goblet, and mast cells under high glucose condition? iii- Is there any systemic factor(s) that is involved in regulation on glucose sensing of*

enterocytes? The studies on these directions will provide better understanding physiological interactions between intestine system and obesity, diabetes.

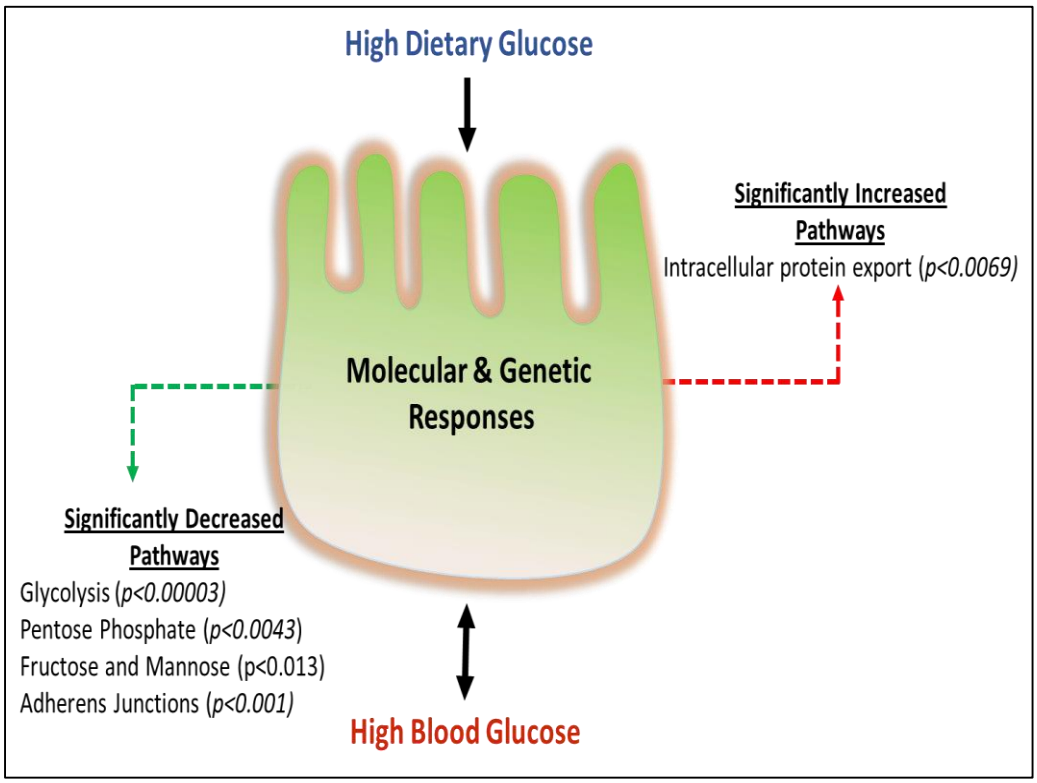

Figure 4: Summary of glucose regulated pathways in fully differentiated human enterocyte cell culture model

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