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Disruption of the GPR35 expression accelerates the formation of foam cells in hyperglycemia

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ABSTRACT

Background: Diabetes is one of the most common metabolic disorders that increase the formation of vascular plaques and foam cells in the vessel wall by increasing inflammation in blood cells and arterial walls. Consequently, understanding the genes involved in this process can greatly help control the progression of vascular diseases.

Methods: In this study, to investigate the response of GPCR receptor that may play a role in the process of foam cell formation and inflammation in diabetes, related RNAseq and microarray data were examined. Also, the association of candidate gene expression with the foam cell formation genes and co-regulating miRNAs, was evaluated.

Results: The results showed that hyperglycemia led to inflammation and decreased *GPR35* gene expression. *GPR35* expression is also reduced in the blood of prediabetic and diabetic patients, and this decrease in expression is accompanied by changes in other genes involved in the process of foam cell formation. Also, three miRNAs named hsa-miR-320c, hsa-miR-183 and hsa-let-7e are co-regulators of *GPR35* gene expression and *CD36, OLR1, ABCG1 and ANGPTL4* genes involved in the foam cell formation.

Conclusion: The present results indicate that the *GPR35* gene may play a role in the process of inflammation and plaque formation in the arteries and it is possible that a decrease in the expression of the *GPR35* gene will accelerate the occurrence of vascular complications of diabetes.

Keywords: GPCR, Diabetes, Foam cell, *GPR35*, Hyperglycemia

Introduction

Diabetes, the most common, chronic metabolic disorder, characterized by hyperglycemia, leads to a wide range of micro- and macrovascular complications in patients (1). More than 60% of deaths due to diabetes are due to cardiovascular complications of diabetes (2). Inflammation is one of the key factors in the development of vascular complications of diabetes. Indeed, persistent increases in blood glucose and advanced glycation end products (AGEs) are associated with the activation of inflammatory pathways such as NF-κB, and Mitogen-Activated Protein Kinases (MAPKs) and DNA damage (3). This activation leads to the production of proinflammatory cytokines (such as TNF-α, IL-6 and IL-1β) and inflammatory factors (such as CRP) that directly or indirectly contribute to the pathogenesis of diabetic complications (4).

The presence of plaque in the vessel is a characteristic feature of atherosclerosis. Vascular plaques are composed of necrotic cell nuclei and macrophages. In the early stages of atherosclerosis, permeability to oxidized low-density lipoprotein (oxLDL) increases, followed by inflammation in arterial endothelial cells. In this process, circulating monocytes and several types of resident tissue cells invade and infiltrate the vascular intima, and monocytes then differentiate into different macrophage subtypes at that site (5,6).

In fact, monocytes and macrophages, as key cells of the immune system, undergo functional and structural changes in response to inflammatory stimuli and hyperglycemic environments. These cells themselves transform into foam cells by absorbing oxidized lipids and accumulating fat in their cytoplasm. The formation of foam cells is

accompanied by increased secretion of cytokines and decreased phagocytosis, which ultimately leads to increased fat accumulation in the arterial wall and contributes to the development of atherosclerotic plaques (5,7). In addition, diabetes contributes to the formation of foam cells by disrupting lipid metabolism and reducing cholesterol efflux from macrophages (8,9). Therefore, investigating the genes that regulate foam cell formation in hyperglycemic conditions could be a step towards better understanding and controlling the progression of atherosclerosis.

G protein-coupled receptors (GPCRs) represent promising therapeutic targets in diabetic atherosclerosis due to their critical involvement in regulating insulin secretion, lipid metabolism, modulating inflammatory cytokine secretion, and influencing macrophage polarization. Also, GPCRs have an important impact on monocyte adhesion, all of which are central to the development and progression of vascular inflammation and atherosclerotic plaque formation in diabetes (10,11).

These seven-fold transmembrane receptors respond to a wide range of inflammatory factors and are involved in the regulation of many intracellular processes so, more than 30% of FDA-approved drugs target GPCRs (12). Previous studies have shown that some GPCRs play important roles in various inflammatory processes and macrophage differentiation. for example, it has been reported that activation of the GPR55 gene plays a significant role in the progression of atherosclerosis (13). Accordingly, clarifying the functional roles of GPCR may provide new therapeutic insight into inflammatory disorders.

So, GPCR may have a strong impact on the regulation of diabetic complications, we selected GPR35 as an inflammatory GPCR receptor that has the highest response to high blood glucose and plays a crucial role in regulating foam cell formation based on analysis of RNA-seq and microarray data. Finally, the response of this gene to hyperglycemia and inflammation treatment in monocyte cells was assessed.

Methods

Bioinformatics studies

To investigate the effect of hyperglycemia on the induction of inflammation in monocytes and its impact on the differentiation of monocytes into macrophages, the GEO dataset was searched for the keywords "diabetes," "monocyte," and "foam cell." The GSE201105, GSE156061 and GSE26168 datasets were selected. Initially, the GSE201105 dataset was analyzed. Normalized count data were extracted and after examining the quality of the data, gene expression changes were examined using the DEseq2 package. So, a heat map of inflammatory GPCRs was drawn via the Pheatmap package. Also, a correlation heat map of the expression of the candidate receptor with genes related to the formation of foam cells was drawn using the correlation (COR) package. Finally, a Gene Enrichment study was performed using the Enrichr website (14) and the FunRich software (15). Also, the GSE26168 dataset was analyzed using the limma package to examine the miRNAs and RNAs that were differentially expressed in the blood of diabetic patients.

Gene-miRNA interaction

The possibility of miRNA binding to the candidate GPCR and genes related to foam cell formation was investigated via the online software miRWalk (16). Gene–miRNA interactions with a score higher than 0.9 were extracted, and then the miRNA-mRNA network was plotted using Cytoscape software (17).

Cell culture

To investigate the effect of hyperglycemia and inflammation on the expression of the candidate receptor in monocytic cells, THP-1 cells were purchased from Pasteur Cell Bank and cell culture was performed in RPMI 1640 medium with 10% FBS (Gibco) and 1% penicillin and streptomycin (100 units/ml penicillin and 100 µg/ml streptomycin). THP-1 cells were maintained in a 37°C incubator containing 5% CO_2. 25 mM glucose is commonly used to mimic hyperglycemic conditions, this concentration effectively induces inflammatory cytokine expression relevant to diabetic conditions (18). Also, 5mm d-glucose is used as a normoglycemic control because it approximates physiological fasting blood glucose levels in humans (19). So, THP-1 cells were cultured in 6-well plates and 25 mM and 5 mM D-Glucose concentrations were used for hyperglycemia and control groups' treatment, respectively.

Primer Design

To assess the expression change of the candidate gene in response to hyperglycemia, specific primer design was performed using Oligo 7 software, so the specificity of the primers was examined using the Primer Blast website. Table 1 lists the sequences of the primers used in this study.

Table 1: Sequence of primers used in this study to assess gene expression by the qPCR method

Gene Symbol	Gene ID:	Sequences	Amplification length(bp)
GPR35	NM_001195381.3	F: 5′ TGGCCAGGTGAGCTGGTTCTG 3′ R: 5′AAGCCAGGACTCTCTGTGCGTG 3′	318
ACTINβ	NM_001101.5	F: 5′ACCCAGCACAATGAAGATCAAGA 3′ R: 5′ACAGTCCGCCTAGAAGCATTTG 3′	175

RNA extraction and cDNA synthesis

To evaluate the expression changes of candidate GPCR in hyperglycemia condition, RNA extraction was performed using RNX Plus solution (Sinaclone, Iran) according to the provided protocol after 48h. In the next step, the quantity and quality of extracted RNA were examined by spectrophotometry and electrophoresis. The cDNA synthesis reaction was performed via the Easy cDNA synthesis kit (Pars Toos Company, Iran) using 3 µg of extracted RNA. Finally, the cDNA synthesis validation was examined by PCR of β-*ACTIN* as the internal control gene.

Gene expression changes by qPCR

Changes in candidate GPCR expression level were evaluated by qPCR method. 10 ng cDNA and 4 pM of each forward and reverse primers and 2 µl of Eva Green (5X, Solis BioDyne, Estonia) in a final volume of 10 µl were used to perform the qPCR reaction. The internal control gene β-*ACTIN* was used to normalize the expression of the candidate gene (20), and the $2^{-\Delta\Delta CT}$ formula was used to estimate the expression fold change (21). This study was performed with at least two biological replicates and two technical replicates.

Statistical analysis

GraphPad Prism software (version 9) was used to draw graphs and perform statistical analysis. For this purpose, an unpaired t-test was used in 2 groups for statistical analysis. The results for each group are shown with mean and standard deviation (mean ± standard deviation), and the significance level was considered to be P-value $\leq$ 0.05.

Results

Selection of GPCR gene by bioinformatic analysis

To find GPCRs that are responsive to hyperglycemia and involved in foam cell formation, the GSE201105 and GSE156061 datasets were analyzed. Boxplots and fireplots show that the data were properly normalized and analyzed. During the differentiation of monocytes into foam cells that were treated with glucose and oxLDL, the number of 2587 genes showed down regulation and 3429 genes were up-regulated. The heatmap of GPCRs related to inflammatory pathways was plotted. Among these, the *GPR35* gene (logFC: -3.099, p-value: 3.07e-26), *ACKR3* (logFC: -5.028, p-value: 7.98e-98), *CNR1* (logFC: -7.84, p-value: 5.58e-4), *LPAR1* (logFC: -8.88, p-value: 2.42e-32) and *CCR7* (logFC: -1.5, p-value: 1.37e-8) showed a significant decrease in expression during foam cell formation in hyperglycemia conditions. *GPR35* gene was selected for further datasets; due to it has similar decreased expression in other blood data of diabetic patients (Figure 1). Also, gene enrichment study showed that the main pathways altered during diabetes and foam cell formation were the interferon gamma, IL3 and ErbB pathways. Also, pathways related to NECTIN junction, integrins and endothelins are among the most important pathways involved during glucose and oxLDL treatment. The results showed that biological processes such as response to interferon and cholesterol, cell junctions by junction molecules and regulation of mast cell activation are among the most important processes affected during the differentiation of monocyte cells into foam cells in hyperglycemia. In addition, the results showed that cytokine responses are among the most important molecular functions associated with the total of altered genes (Figure 2). According to data extracted from the DisGeNET website, the total of altered genes is significantly associated with a wide range of inflammatory diseases, including acute coronary syndrome, arthritis, coronary arteriosclerosis and atherosclerosis. Therefore, to confirm the RNAseq data, changes in *GPR35* expression in response to hyperglycemia were examined in THP-1 monocyte cells.

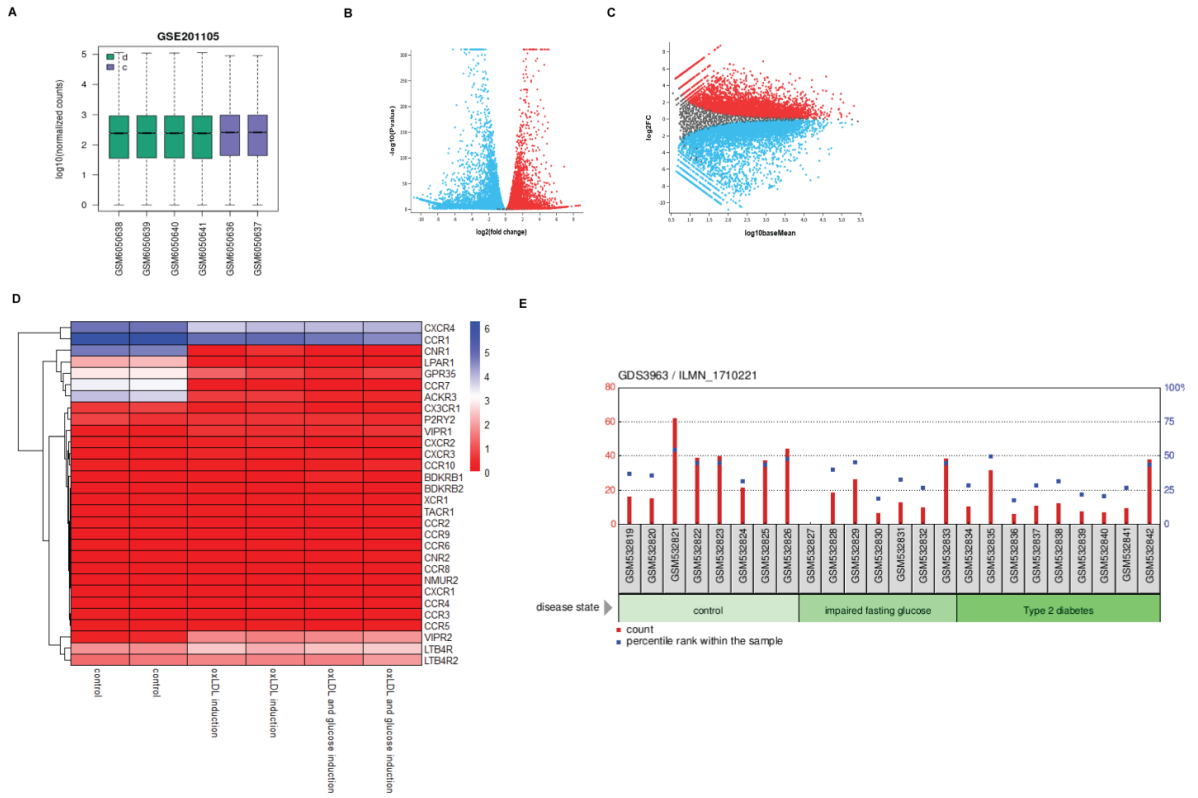

Figure 1: Bioinformatic studies for gene selection: To select GPCR genes involved in the differentiation of monocytes into foam cells, the GSE201105 dataset was analyzed. A: Box plot. B: Flame plot. C;Gene expression change plot of the GSE201105 dataset. D; Heatmap of inflammatory GPCR genes expression in the GSE201105 dataset. E;The reduction of *GPR35* gene expression was also confirmed in the blood of diabetic and prediabetic patients via GSE26168 dataset microarray data.

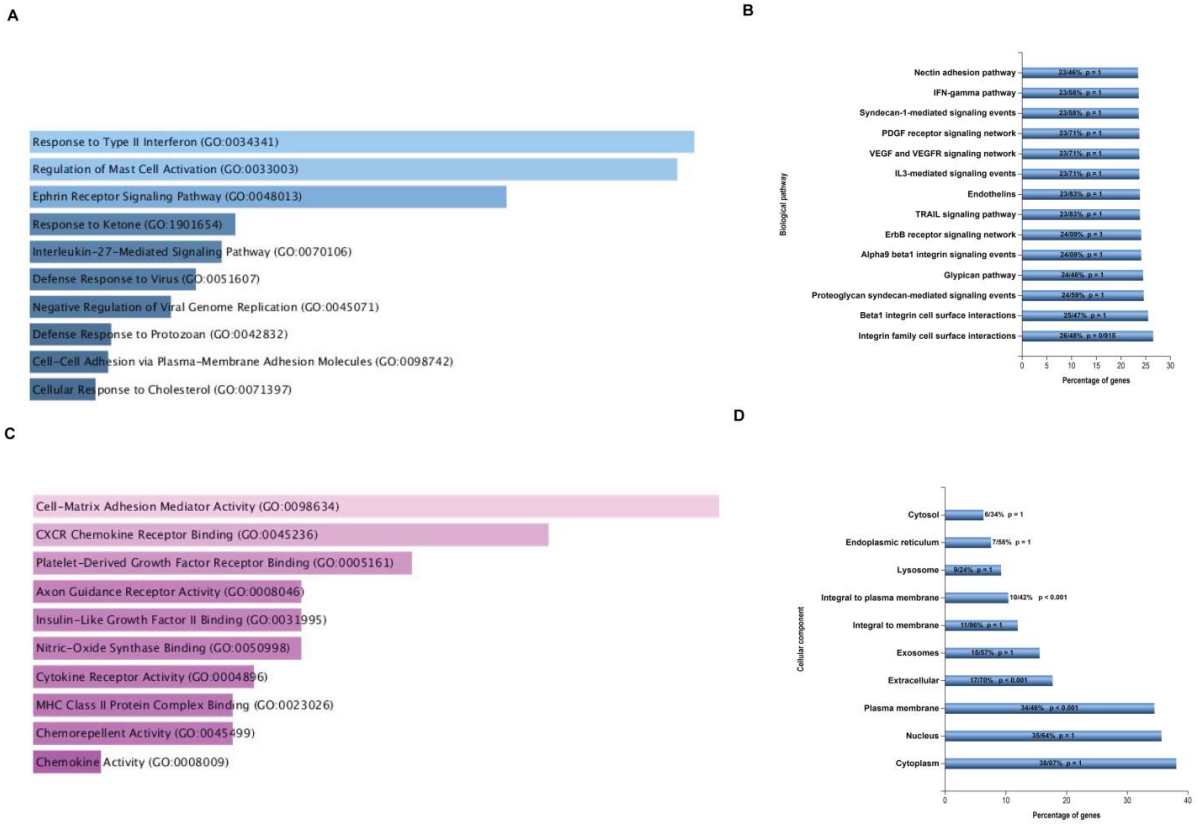

Figure 2: Gene enrichment plot related to differentially expressed genes during foam cell formation by glucose and oxLDL treatment. A: Shows biological processes. B: shows cellular pathways. C: Shows molecular function. D: Shows cellular components.

Correlation between the GPR35 gene and other genes involved in the foam cell formation process

The correlation between *GPR35* gene expression and the expression of the most important genes involved in the foam cell formation process has been shown by drawing the expression co-occurrence heatmap during glucose and oxLDL treatment. Of 28 genes involved in the foam cell formation process, 4 genes named *ABCG1*, *OLR1*, *ANGPTL4* and *CD36* showed significant expression changes in the blood of diabetic patients compared to healthy individuals and during the differentiation of monocyte cells into foam cells. The decreased expression of the *GPR35* gene is positively correlated with the *ANGPTL4* and *ABCG* genes, which have a protective role in the foam cell formation process, and the decreased expression of *GPR35* is negatively correlated with the *CD36* and *OLR1* genes, which accelerate foam cell formation. To investigate the common regulatory factors of these genes with the *GPR35* gene, miRNA expression changes were examined in the blood of diabetic patients. 8 miRNA genes showed significant expression changes. In the next step, sponging these miRNAs via the *GPR35* receptor and *ABCG1*, *OLR1*, *ANGPTL4* and *CD36* was investigated via the miRWalk dataset. Three miRNAs hsa-miR-320c, hsa-miR-183 and hsa-let-7e target the *GPR35* gene. The results showed that there is a possibility of a common miRNA-based regulatory mechanism between the *GPR35* gene and genes involved in the process of foam cell formation (Figure 3). The number of binding sites and the energy of each binding are listed in Table 2.

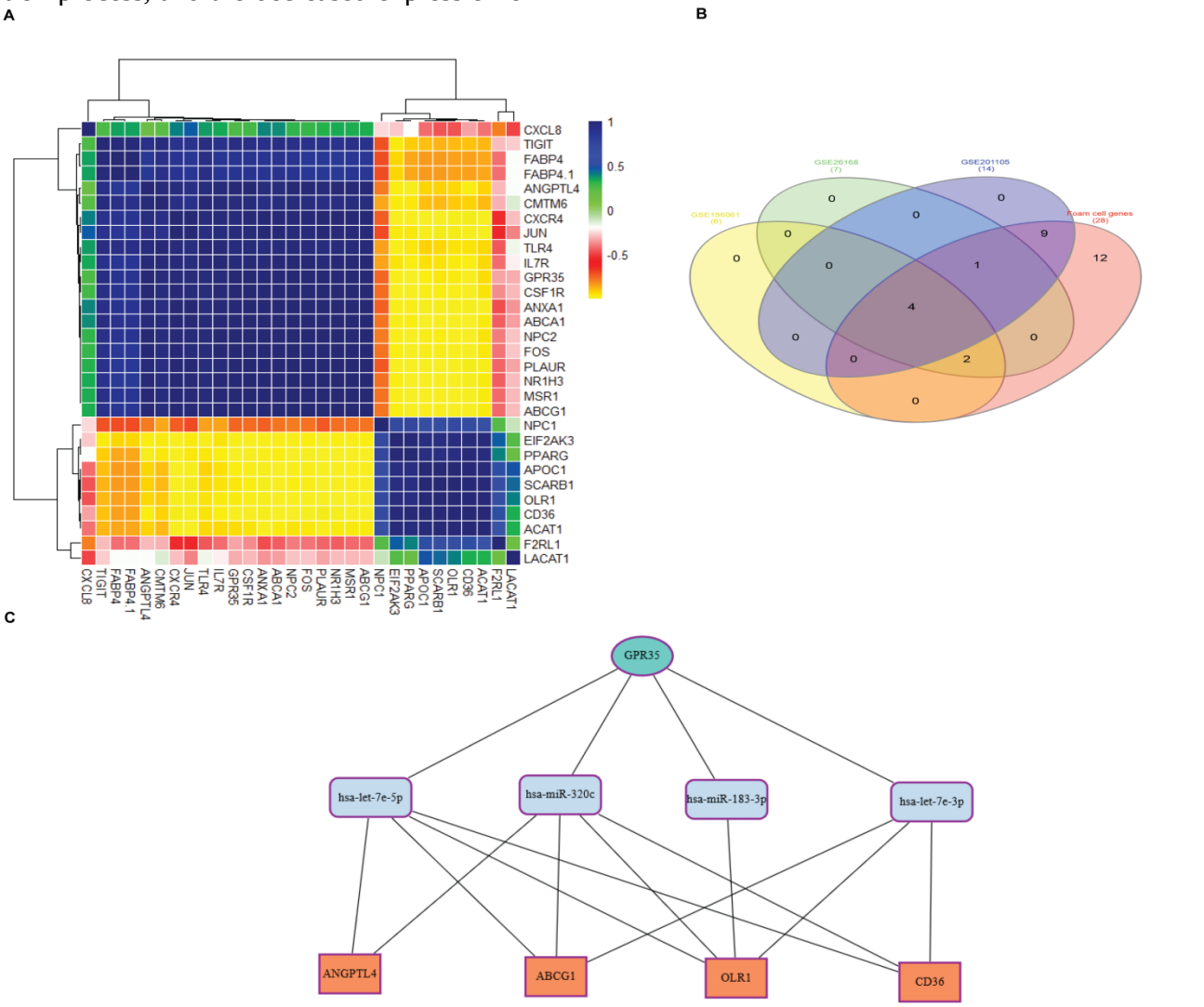

Figure 3: Bioinformatics studies showed that the *GPR35* gene is associated with a set of genes involved in the process of foam cell formation. A: Venn chart showing the commonalities of the altered expression genes involved in the process of foam cell formation in the blood of diabetic patients and the data of monocyte cell differentiation into foam cells. B: Correlation -expression heatmap of foam cell formation related genes with the *GPR35* gene. C: Possible regulatory interaction between the *GPR35* gene and genes involved in the process of foam cell formation by miRNA.

Table 2: Similar miRNAs can target *GPR35* gene and some genes related to foam cell formation. The binding site, number of bindings, and binding energy of each miRNA to the target gene are listed in the table.

miRNA ID	genesymbol	Binding	energy	position	Number of site
hsa-let-7e-3p	*GPR35*	0.923077	-21.2	3UTR	1
hsa-let-7e-3p	*CD36*	1	-19.1	3UTR	1
hsa-let-7e-3p	*ABCG1*	1	-20.7	CDS	4
hsa-let-7e-3p	*OLR1*	0.961538	-21.5	5UTR	1
hsa-let-7e-5p	*GPR35*	0.923077	-25.1	5UTR	1
hsa-let-7e-5p	*CD36*	0.923077	-17.3	CDS	3
hsa-let-7e-5p	*OLR1*	0.923077	-18.5	3UTR	3
hsa-let-7e-5p	*ABCG1*	1	-23.3	CDS	4
hsa-let-7e-5p	*ANGPTL4*	0.923077	-21.7	CDS	2
hsa-let-7e-5p	*ANGPTL4*	1	-21.8	CDS	1
hsa-miR-183-3p	*GPR35*	0.923077	-22	3UTR	2
hsa-miR-183-3p	*OLR1*	0.923077	-21	3UTR	6
hsa-miR-320c	*GPR35*	0.923077	-22.9	3UTR	1
hsa-miR-320c	*GPR35*	1	-22.9	3UTR	1
hsa-miR-320c	*CD36*	0.923077	-20.9	5UTR	2
hsa-miR-320c	*CD36*	0.923077	-19.5	CDS	3
hsa-miR-320c	*OLR1*	0.923077	-25.2	3UTR	1
hsa-miR-320c	*OLR1*	1	-25.2	3UTR	3
hsa-miR-320c	*OLR1*	0.923077	-22.8	5UTR	1
hsa-miR-320c	*OLR1*	0.923077	-23.4	CDS	1
hsa-miR-320c	*OLR1*	1	-23.4	CDS	2
hsa-miR-320c	*ANGPTL4*	0.923077	-20.8	CDS	1
hsa-miR-320c	*ABCG1*	0.923077	-22.9	3UTR	1
hsa-miR-320c	*ABCG1*	0.923077	-22.9	3UTR	3
hsa-miR-320c	*ABCG1*	1	-21	CDS	5
hsa-miR-320c	*ANGPTL4*	0.923077	-20.8	CDS	1

Hyperglycemia reduced the GPR35 gene expression in THP1 cells

To assess the effect of hyperglycemia on GPR35 gene expression based on a previous study, hyperglycemia was induced by 25 mM d-glucose concentration and for normal conditions, 5 mM d-glucose was used (22). The results of qPCR analysis showed hyperglycemia decreased *GPR35* mRAN expression by about a 0.03-fold in THP1 cells with a P-value: 0.0001 after 48h (Figure 4A).

TNF treatment decreased the GPR35 gene expression in THP1 cells

According to previous studies, the level of cytokine factors like TNFα increases in atherosclerosis and leads to increased vascular inflammation (23). Therefore, to evaluate the effect of TNFα treatment on *GPR35* gene expression, THP-1 monocyte cells were treated with a 1nM TNFα for 24 hours. The results showed that TNFα treatment decreased the *GPR35* mRNA expression by about 0.5-fold with a P-value: 0.0107 (Figure 4B).

Discussion

In diabetes, the rapid progression of atherosclerosis goes beyond conventional vascular risk factors such as dyslipidemia and hypertension. In diabetes, hyperglycemia, oxidative stress, and inflammation play pivotal roles in the development of vascular diseases. Diabetes accelerates the formation of vascular plaques and atherosclerosis (9). According to studies, enhanced blood glucose levels increase the uptake of oxidized LDL and lipids in monocyte and macrophage cells by changing the expression of some receptors such as CD36, thereby increasing the formation of foam cells in the vascular artery wall (8,24).

Biological pathways such as cellular response to cholesterol and lipids, changes in cell-to-cell connections like endothelin intraction, alpha and beta integrins, and cytokine responses altered during foam cell formation. From these, integrins play a significant impact on lipid uptake and foam cell formation process. According to previous studies, the expression of integrins such as αVβ3 increases in macrophages in early and advanced atherosclerotic lesions (25). Also,

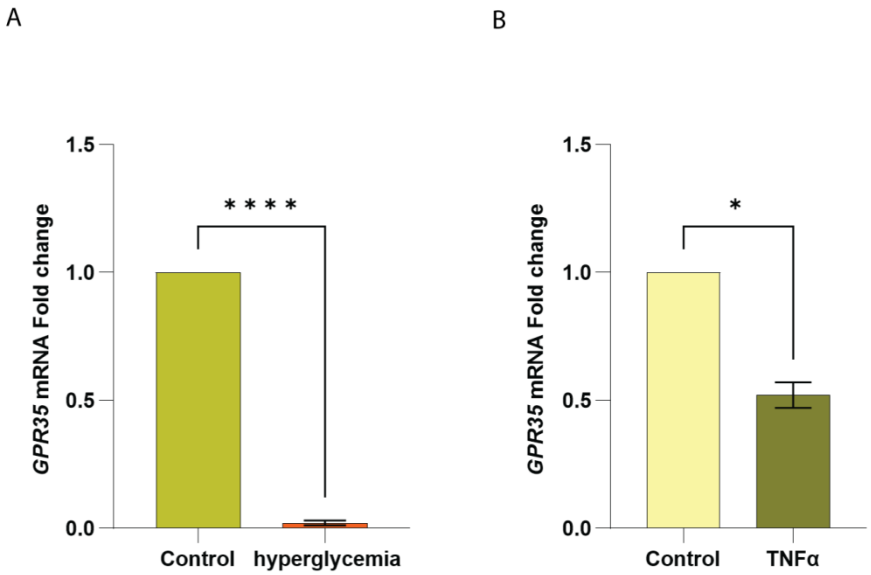

Figure 4: Hyperglycemia and TNFα treatment reduced *GPR35* mRNA expression in THP-1 monocytic cells. A: GPR35 mRNA response to hyperglycemia induction. B: GPR35 mRNA response to TNFα treatment. Changes in *GPR35* mRNA expression were measured by q-PCR. The internal control gene *β-ACTIN* was used to normalize the changes in candidate gene expression. *P-value = 0.03, **P-value: 0.0107.

changes in endothelin-related pathways lead to activation of endothelial cells, recruitment of monocytes, and increased lipid uptake and ultimately the formation of foam cells (26). The totality of cellular pathways altered in RNAseq data analysis indicated that inflammatory pathways associated with cytokines, including chemokine inflammatory receptor, play an important role in this process. GPCRs are one of the most important inflammatory receptors.

Since GPCRs in diabetic patients can respond to a wide range of metabolites in the blood and lead to changes in the level of intracellular inflammation and lipid uptake, this study focused on the changes in the expression of inflammatory GPCRs (27). The present result showed that, oxLDL and glucose treatment during the process of foam cell formation led to a decrease in the expression of the *GPR35* gene. This gene is located on the long arm of chromosome 2, which is one of the diabetes hotspots, and the haplotype present at this chromosomal locus can be associated with susceptibility to diabetes. Genome-wide association studies (GWAS) have identified several single-nucleotide polymorphisms (SNPs) in or near the *GPR35* gene that are associated with an increased risk of developing type 2 diabetes, especially in specific populations such as Mexican-Americans. Some SNPs are associated with type 2 diabetes, although in some cases, adjacent genes such as *CAPN10* may be more directly involved in the susceptibility to the disease (28–31). Gene expression association studies have also shown that Ser294Arg variant in the *GPR35* gene is associated with coronary artery calcification in cardiovascular patients (32). The presence of genetic variants associated with increased diabetes incidence and altered *GPR35* gene expression in the blood of diabetic patients and foam cell formation may indicate the impact of *GPR35* dysfunction in increasing the likelihood of diabetes and increasing the incidence of vascular complications of diabetes.

GPR35 expression is reduced in blood monocyte cells of prediabetic and diabetic patients. It can be suggested that the decreased expression of GPR35 indicates a change in the *GPR35* function in blood cells in response to hyperglycemia. Also, TNF treatment reduced *GPR35* expression in THP-1 cells, which showed *GPR35* response to an inflammatory condition like the chronic phase of diabetes. Since high blood glucose levels lead to increased inflammation and some cytokines, there is a possibility that the GPR35 gene responds to cytokine factors.

Therefore, to understand whether this decreased expression is a protective response of monocyte cells to hyperglycemia or an aggravating response, co-expression analysis studies were performed. The results showed that the decreased *GPR35* expression is positively associated with the *ABCG1* and *ANGPTL4* genes

that protect against foam cell formation. In contrast, the decreased *GPR35* expression is negatively associated with the *CD36* and *OLR1* genes that play an accelerating role in foam cell formation. In this line, it may be concluded that hyperglycemia causes a stable decrease in *GPR35* gene expression by increasing inflammation, followed by the enhancement of the foam cell formation pathway. The existence of an expression correlation between the *GPR35* gene and some genes involved in foam cell formation may show the existence of epigenetic factors and common transcription factors between these genes.

miRNAs are one of the most important factors regulating gene expression. Three miRNAs named hsa-miR-320c, hsa-miR-183 and hsa-let-7e showed significantly increased expression in blood monocytes of diabetic patients; based bioinformatics study, these miRNAs can target the GPR35 gene. The enhanced expression of the two miRNAs hsa-miR-320c and hsa-miR-183 likely increases the incidence of diabetic complications by affecting downstream targets. Studies have shown that these two miRNAs increase the formation of foam cells (33,34). MiR-183 increases the activation of pre-atherogenic macrophages. Inhibition of miR-183 reduces the formation of foam cells by increasing cholesterol efflux and reducing cholesterol uptake in macrophages. It reduction also changes the polarity of macrophages from the pro-inflammatory M1 phenotype to the anti-inflammatory M2 phenotype, which helps to resolve inflammation (34).

The miR-320 family has similar sequences and functions. miR-320b, which is closely related to miR-320c, was reported to suppress macrophage cholesterol efflux by targeting the ATP-binding cassette transporter (ABCG1) and increase lipid accumulation in macrophages and foam cell formation, thereby promoting atherosclerosis (35). Given the similarity, miR-320c is likely to have comparable effects on lipid metabolism and plaque progression. Also, miR-320 can directly target the *CD36* gene promoter and increase its expression (36). Therefore, it may be suggested that reducing *GPR35* gene expression increases miR-320 binding to the *CD36* gene promoter and may increase *CD36* gene expression, ultimately contributing to increased foam cell formation during hyperglycemia. On the other hand, increased expression of miR-183 accelerates the inflammatory process during diabetes by targeting genes that suppress foam cell formation. Possibility, decreased expression of the *GPR35* gene correlates with increased the access of mir-183 to its other targets. In contrast, it has been suggested that the hsa-let-7e family can play a role in reducing lipid accumulation and foam cell formation, which may indicate the protective role of this mirRNA during hyperglycemia (37). However, based on data extracted from the miRWalk database, the number of binding sites of this miRNA for protective genes is higher and it has fewer binding sites for *CD36* and *OLR1* genes.

Therefore, it may be stated that the total interaction of the *GPR35* gene with genes involved in the formation of foam cells can play a role in the regulation of this pathway through some non-coding RNAs, and the regulation of the *GPR35* gene can play a role in the occurrence of diabetic complications. Some experimental studies are needed to validate the functional impact of the *GPR35* gene in foam cell formation. For example, *GPR35* knockdown in hyperglycemic conditions can clarify the functional role of the GPR35 gene in foam cell formation in monocyte cells. Also, strong validation of the impact of this gene can be obtained by rescue experiments via GPR35 agonist or overexpression of *GPR35*. Investigation of GPR35 expression and cell distribution in the foam cell formation process can clarify the best pattern of GPR35 response to hyperglycemia and inflammatory cytokines.

Conclusion

Hyperglycemia accelerates the formation of foam cells in the arterial wall by increasing inflammation and disrupting the flow of lipid accumulation. Inflammatory GPCR receptors can play an important role in regulating this process. The *GPR35* receptor expression decreased in the blood of prediabetic and diabetic patients. Also, this decrease in expression will continue in the process of foam cell formation in hyperglycemia conditions. Probably, the decreased expression of *GPR35* is accompanied by the activation of foam cell formation genes and the inhibition of protective genes. In toto, the role of factors such as non-coding RNAs in regulating this process can be very important. Overall, this study

demonstrates the importance of the *GPR35* gene for further research to understand the molecular mechanism of diabetes and vascular plaque formation.

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Conflict of interest statement

The authors declare that there are no conflicts of interest related to this study

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