Activation of Wnt signaling reduces high-glucose mediated damages on skin fibroblast cells

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A R T I C L E I N F O	A B S T R A C T
	Objective(s): High-glucose (HG) stress, a mimic of diabetes mellitus (DM) in culture cells, alters expression of a large number of genes including Wnt and NF-κB signaling-related genes; however, the role of Wnt signaling during HG-mediated fibroblast damage and the relationship between Wnt and NF-κB signaling have not been understood. In this study, we aimed to investigate the ffects of Wnt signaling on HG-mediated damages. *Materials and Methods:* Wnt3a was treated to HG-stressed human primary foreskin fibroblasts and the levels of Wnt signaling markers and cell proliferation were monitored. In addition, Wnt3a and NF-κB signaling inhibitor were assisted to analyze the relationship between two pathways. *Results:* The results indicated that HG treatment repressed β-catenin level, and Wnt3a treatment increased the levels of β-catenin and *FZD8* as well as cell proliferation. RNA-seq based transcriptome analysis identified 207 up-regulated and 200 down-regulated genes upon Wnt3a supply. These altered genes are distributed into 20 different pathways. In addition, gene ontology (GO) analysis indicates that 20 GO terms are enriched. Wnt signaling genes were further verified by qRT-PCR and the results were similar with RNA-seq assay. Since NF-κB signaling negatively regulates Wnt marker gene expression, Bay117082, a typical NF-κB signaling inhibitor and Wnt3a were supplemented for testing β-catenin and phosphorylated IκBα (p-IκBα), respectively. *Conclusion:* HG positively inhibits Wnt signaling, and signaling activation via supplementation of Wnt3a rescued the defect caused by HG. NF-κB signaling negatively regulates accumulation of β-catenin, but Wnt signaling has no effects on IκBα activation.

Introduction

Diabetes mellitus (DM), defined as high blood sugar concentration, is the severe metabolic diseases, and its associated complications affect a large number of people in the developed world. The typical symptom of DM is unfit hyperglycemia, which causes severe diabetic complications including heart, kidney, and skin damages. A large number of patients suffering from diabetic complications showed difficulties in skin wound healing (1). High-glucose (HG; hyperglycemia) induces defects in angiogenesis. Blood resupply in the damaged tissues is important for recovery of skin ulcer in patients with DM (2).

Skin ulcer repair needs demand of recruitment of several cell types including keratinocytes, fibroblasts, endothelial cells, macrophages and platelets. Fibroblast is one of the important cell layers whose proliferation and migration, collagen deposition and remodeling, wound contraction, and angiogenesis are the necessary process for wound repair. Extracellular matrix (ECM) forms the largest component of the dermal skin layer (3). Fibroblast is mainly participated in producing and remodeling the ECM, and it is also important for the formation of granulation tissue and subsequent skin repair (4, 5). Reactive oxygen species (ROS) are known as a secondary messenger, and its level inside the cell regulates cell proliferation, maturation and differentiation, the key steps of wound healing (6). Fibroblasts in patient with diabetic ulcer are usually large and widely spread in *in vitro* culture condition compared with the normal fibroblasts in age- matched controls. They often show abnormal endoplasmic reticulum, increased number of vesicular bodies and lost microtubular structure. Therefore, DM affects protein turnover, autonomous trafficking and normal protein secretion in diabetic ulcer fibroblasts (7, 8). Fibroblasts from diabetic ulcer induce defect in cell proliferation that may result in a decrease of ECM protein production and further delayed wound healing (7). HG-induced fibroblast migration was identified as a result of

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reduction of JNK activity (9). Previous transcriptome study identified that HG alters expression of a large number of genes including genes associated with inflammatory response and Wnt signaling (10).

The canonical Wnt signaling pathway, also defin-ed as the Wnt/β-catenin or the β-catenin/T-cell factor (TCF) pathway (11), regulates diverse aspects of biological processes (12-14). Hallmark of the Wnt/β-catenin pathway is the stabilization of cyto-solic β-catenin. Under unstimulated conditions, β-catenin is constantly phosphorylated by a destruct-tion complex consisting of glycogen synthase kinase-3β (GSK3β) and other proteins (15); phosphorylated β-catenin is ubiquitinated by this complex and targeted for degradation by the proteasome (15, 16). Activation of the Wnt cascade inhibits GSK3β activity, allowing β-catenin to accumulate and enter the nucleus, where it associates with T-cell factor/lymphoid enhan-cer binding factor (TCF/LEF), leading to the transcript-tion of Wnt signaling genes that participate in cell survival, proliferation, and differentiation (15). However, the role of Wnt signaling in DM-mediated skin fibroblast damage has not been reported.

Here, the Wnt signaling effects were analyzed by exogenous supply of Wnt3a to HG-stressed skin fibroblast cells, and the Wnt signaling marker changes and cell proliferation were monitored. Furthermore, RNA-seq assay was performed to identify Wnt3a in transcriptome changes, and the relationship between NF-κB and Wnt signaling was tested. This study provided useful information for understanding molecular mechanism behinds HG-induced damages on skin fibroblasts, and the results may be important for DM-induced skin ulcer therapy.

Materials and Methods

Human foreskin fibroblast cell culture

The human fibroblast cells were isolated and subsequently cultured for testing high glucose effects. All the procedures for purification and culture were followed the protocol as described (9). Human foreskin samples were collected from 6 patients in Department of Dermatology, the First Affiliated Hospital, Wenzhou Medical University (Wenzhou, China). This study was approved by the ethics committee of Wenzhou Medical University (Wenzhou, China) and written informed consent was obtained from all the patients involved. HG and Wnt3a were treated to the fibroblast cells for 1 or 3 hrs for Western blot, qRT-PCR, and cell proliferation assays.

Cell proliferation assay

Cell proliferation ability was analyzed by using a CCK-8 Kit (Dojindo Bio., Japan). The fibroblast cell culture and measurement of cell densities under HG (50 mM) and Wnt3a (100 ng/ml) treatments were followed as described (9).

RNA deep sequencing

Total RNA extracted from human foreskin fibro-blasts cultured in 5.5 mM concentration of glucose containing medium with or without Wnt3a (100 ng/ml, 1 hr) for the RNA-seq experiments. RNA-Seq experiments and data analysis were performed by the Novel Bioinformatics Co., Ltd. (Shanghai, China, http://www.novelbio.com/). The RNA-seq data is deposited in a personal hard disk and available to share upon requested.

Analysis of the pathway and GO category

Differentially expressed genes were identified from statistical analysis testing for association with biological process gene ontology (GO) terms (17). Fisher's exact test was used to classify the GO category, and the false discovery rate (FDR) was used to calculate and correct the *P*-value (18). Enrichment of GO members among differentially expressed gene sets was found using the one-tailed Fisher's exact test for 2 × 2 contingency tables (19), and it provides a measure of the significance of the function that as the enrichment increases, the corresponding function is more specific and helpful for finding GOs with a more concrete function description in the experiment.

Pathway analysis was utilized to identify the significantly changed pathway of the differential genes according to KEGG, BioCarta, and Reatome databases. Fisher's exact test has been followed by Benjamini–Hochberg (BH) multiple testing correction to select the significant pathway, and the significance between groups was defined by *P*-value and FDR (20).

Total RNA extraction, cDNA synthesis and qRT-PCR

Total RNA was extracted from the fibroblasts of stimulated or un-stimulated with high concentration of glucose (30 mM) and Wnt3a (100 ng/ml, Abcam, Cat no. ab23327, USA). The cell monolayer was rinsed with ice cold phosphate buffered saline (PBS) once. Then, the cells were directly lysed in a culture dish by addition of 1 ml of Trizol Reagent (Trizol Reagent, Invitrogen) per each 3.5 cm diameter dish, and then 0.2 ml of chloroform was added per 1 ml of Trizol Reagent. 2 μg of total RNA was reverse-transcribed to cDNA by using a GoScript Reverse Transcription Kit (Reverse Transcription System, Promega) following the manufacturer's instructions. The transcript levels were normalized against that of *GAPDH*. Gene specific primer sequences used for qRT-PCR are listed in Table 1.

Western blot analysis

Total proteins were separated on a SDS-PAGE gel after extraction and transferred onto Immobilon-P Transfer Membranes (Millipore, Tokyo, Japan). The membranes were incubated in 1x Tris buffered saline (TBS) containing 5% skim milk and 0.05% Tween-20 for 1-2 hrs and reacted with corresponding primary

Table 1. qRT-PCR primer sequences

Primer	Sequences
Wnt2 F	AAGGTACATTGGGGCACTGG
Wnt2 R	CGCGAGTAATAGCGTGGACT
Wnt3 F	ATCATAAGGGGCCGCCTGGCGAAGGCTGG
Wnt3 R	CTTGCAGGTGTGCACGTCGTAGA
Wnt11 F	GAACTGCTCCTCCATTGAGCTC
Wnt11 R	GGTATCGGGTCTTGAGGTCAG
TCF7 F	CTGCAGACCCCTGACCTCTCT
TCF7 R	ATCCTTGATGCTAGGTTCTGGTGT
FZD8 F	CTGGTGGAGATCCAGTGCTC
FZD8 R	TTGTAGTCCATGCACAGCGT
GAPDH F	GACCTGCCGTCTAGAAAAAC
GAPDH R	CTGTAGCCAAATTCGTTGTC

antibodies at 4 °C overnight. An anti- β-catenin antibody (1:2000, Abcam, Cat no. ab16051, Cambridge, USA), anti-p-IKBα antibody (1:2000, Abcam, Cat no. 39A1431, Cambridge, USA), anti-IKBα antibody (1:2000, Abcam, Cat no. ab7217, Cambridge, USA), and anti-GAPDH antibody (1:2000, Abcam, Cat no. abcam 9484, Cambridge, USA) were used as primary antibodies. The membranes were incubated for 1 hr with an anti-mouse or anti-rabbit horseradish peroxidase (HRP)-linked secondary antibody (1:2000, Cell Signaling Technology, Inc., Cat. no. 7074, Danvers, MA, USA).

Statistical analysis

Statistical analysis was performed with Prism 5 software package (GraphPad, San Diego, CA). Significant differences were expressed as mean±SE. Comparison between two groups was analyzed by student's *t* test, while comparisons between more than two groups were performed by using one-way ANOVA.

Results

HG reduces β-catenin accumulation in skin fibroblasts

Since HG stress changed gene expressions of a large number of genes including Wnt signaling genes (10); therefore, protein level of β-catenin, a key regulator of Wnt signaling, was further analyzed. The results showed that β-catenin level was significantly reduced after 1 hr of HG (30 mM) treatment (Figures 1A, 1B). To test the activation of Wnt signaling by exogenous supply of a recombinant Wnt3a, the fibroblast cells were treated with Wnt3a (100 ng/ml) for 1 and 3 hrs, and β-catenin levels were examined in non-treated (Con) Wnt3a treated cells. The results of Western blot analysis showed that Wnt3a increased the level of β-catenin in 1 and 3 hrs of treatment (Figures 1C, 1D). Also, Wnt3a together with HG treatment for 1 hr inhibited HG effects on β-catenin repression in fibroblasts (Figures 1E, 1F).
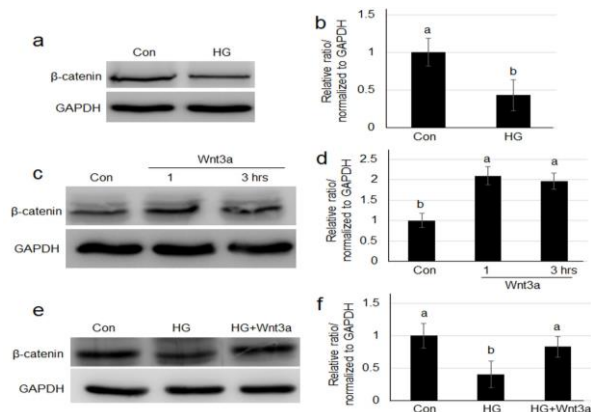

Figure 1. High-glucose (HG) and Wnt3a effects on β-catenin levels in fibroblast cells. (A) Western blot analysis detected β-catenin levels in normal and HG-treated cells (3 hrs). (B) The band density shown in (A) was measured. The experiments repeated three times. (C) β-catenin levels in normal and Wnt3a (100 ng/ml)-treated cells (1 and 3 hrs after treatment) were analyzed. (D) The band density shown in (C) was measured. The experiments repeated three times. (E) β-catenin levels in the cells with HG or HG together with Wn3a treatment for 3 hrs were analyzed. (F) The band density shown in (E) was measured. The experiments repeated three times. Significant differences at *P*-value<0.05 level are indicated by different letters

Wnt3a treatment activated expression of FZD8 and fibroblast cell proliferation

In previous transcriptome study, HG reduced expression of a Wnt-related gene *FZD8* and cell proliferation (10). Therefore, *FZD8* gene expression and cell proliferation were analyzed after treatment of the cells with HG, Wnt3a and HG together with Wnt3a. The results of qRT-PCR showed that HG and Wnt3a treatment for 1 hr repressed and induced *FZD8* gene expression, respectively, while treatment of HG together with Wnt3a exhibited no significant differences from non-treated cells (Con) (Figure 2A). Findings showed that 50 mM glucose obviously inhibited fibroblast cell proliferation (10), but Wnt3a activated cell proliferation of normal and glucose treated (50 mM) skin fibroblast cells (Figure 2B).

Wnt3a dependent transcriptome changes in fibroblasts

To identify Wnt-regulated genes and pathways, RNA-Seq experiments were performed using normal and Wnt3a-treated human fibroblast cells. Since *FZD8* and β-catenin levels were obviously changed after 1 hr of treatment (Figure 1B), the fibroblast cells stimulated for 1 hr with Wnt3a and non-treated cells were collected for RNA-Seq analysis. The RNA-seq results showed that 407 genes were differentially expressed (>1.5 fold change; *P*-value<0.05) in the Wnt3a-treated fibroblasts. Among them, 200 genes were down-regulated, while 207 genes were up-regulated. GO analysis indicated that 20 GO terms were (*P*-value<0.01), stress, and ATP activity (Table 2). Further pathway analysis that was performed with

Table 2. Gene ontology (GO) classification

| GOID	GOTerm	Log2 fold	Expression
GO:0042310	Vasoconstriction	1.81	up
GO:0006950	Response to stress	1.47	up
GO:0071480	Response to gamma radiation	1.54	up
GO:0071277	Response to calcium ion	-.134	down
GO:0033120	Regulation of RNA splicing	-1.45	down
GO:0019065	Receptor-mediated endocytosis of virus by host cell	1.24	up
GO:1903598	Positive regulation of gap junction assembly	1.81	up
GO:0001960	Negative regulation of cytokine-mediated signaling pathway	1.81	up
GO:0032400	Melanosome localization	1.57	up
GO:0043409	MAPK cascade	1.81	up
GO:0009062	Fatty acid catabolic process	-1.10	down
GO:0071207	Histone pre-mRNA stem-loop binding	1.08	up
GO:0006970	Response to osmotic stress	-1.18	down
GO:0051301	Cell division	1.25	up
GO:0045931	Positive regulation of mitotic cell cycle	1.03	up
GO:0070836	Caveola assembly	1.81	up
GO:0006874	Calcium ion homeostasis	1.81	up
GO:0002904	B cell apoptotic process	-1.46	down
GO:0032780	Negative regulation of ATPase activity	2.61	up
GO:0002079	Inner acrosomal membrane	2.04	up
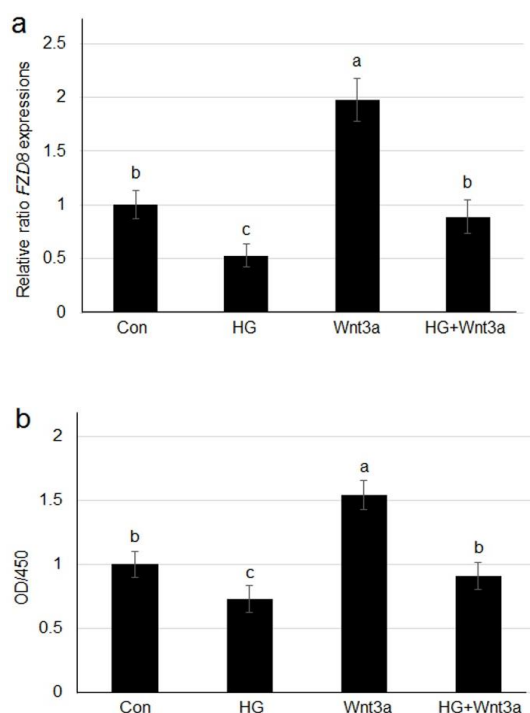
Figure 2. High-glucose (HG) and Wnt3a effects on *FZD8* gene expression and cell proliferation. (A) qRT- PCR was performed to monitor the expression level of *FZD8*. *GAPDH* was used as an internal control. Data represent mean values ± SE of 3 replicates. (B) Cell proliferation before and after the HG and Wnt3a treatment with 10% fetal bovine serum (FBS) in culture medium was measured by CCK-8 assay after a 72-hr of incubation. Significant differences at *P*-value<0.05 level are indicated by different letters
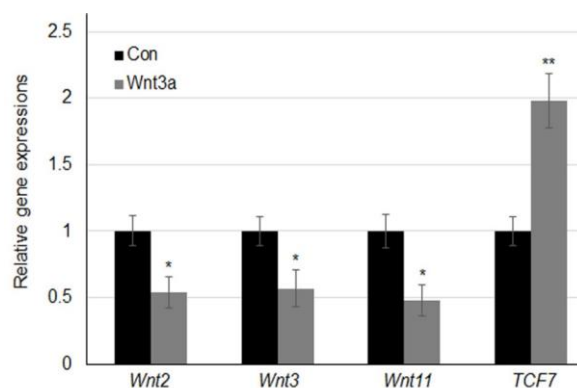

Figure 3. Wnt3a-dependent expression of Wnt signaling genes. qRT-PCR was performed to monitor the mRNA levels of *Wnt2*, *Wnt3*, *Wnt11*, and *TCF7*. *GAPDH* was used as an internal control. Data represent mean values ± the SE (n=6 replicates; *P*-value<0.05, **P*-value<0.01 versus the untreated control group)

differentially expressed genes resulted in 20 different enriched pathways, including Wnt, vascular endothelial growth factor (VEGF), and the transforming growth factor beta (TFG-beta) signaling pathways (Table 3). To verify RNA-Seq results, 4 Wnt signaling related genes (*Wnt3*, *Wnt3*, *Wnt11*, and *TCF7*) were further monitored by qRT-PCR. *Wnt2*, *Wnt3*, and *Wnt11* were suppressed, while *TCF7* was induced by Wnt3a treatment, and the qRT-PCR results were similar with RNA-seq data (Figure 3).

Table 3. Pathway classification

| Pathway ID	PathwayTerm	Log2 fold	Expression
PATH:04310	Wnt signaling pathway	1.39	up
PATH:04370	VEGF signaling pathway	-1.34	down
PATH:04120	Ubiquitin mediated proteolysis	1.58	up
PATH:04350	TGF-beta signaling pathway	1.40	up
PATH:05222	Small cell lung cancer	-1.64	down
PATH:00400	Phenylalanine, tyrosine and tryptophan biosynthesis	-1.14	down
PATH:00512	O-Glycan biosynthesis	1.14	up
PATH:05410	Hypertrophic cardiomyopathy (HCM)	-1.85	down
PATH:03440	Homologous recombination	1.36	up
PATH:05414	Dilated cardiomyopathy	-1.85	down
PATH:00471	D-Glutamine and D-glutamate metabolism	1.19	up
PATH:04060	Cytokine-cytokine receptor interaction	-1.51	down
PATH:04610	Complement and coagulation cascades	-4.92	down
PATH:04710	Circadian rhythm mammal	-1.39	down
PATH:04110	Cell cycle	-1.99	down
PATH:04260	Cardiac muscle contraction	-1.85	down
PATH:05219	Bladder cancer	1.03	up
PATH:04360	Axon guidance	1.24	up
PATH:05412	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	-1.85	down
PATH:00250	Alanine, aspartate and glutamate metabolism	-2.24	down
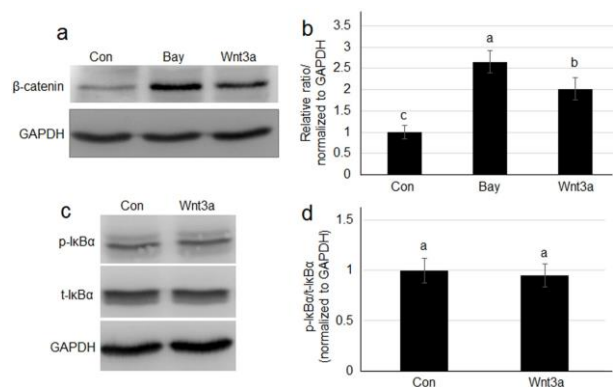
Figure 4. Relationship between NF-κB and Wnt signaling pathways in skin fibroblasts. (A) β-catenin levels were analyzed after Bay117082 (Bay), a typical NF-κB signaling inhibitor, and Wnt3a treatment. (B) The band density shown in (A) was measured. The experiments repeated three times. (C) Western blot analysis was performed to analyze the levels of p-IκBα and total IκBα (t-IκBα) after Wnt3a treatment for 1 hr. (D) The band density shown in (C) was measured. The experiments repeated three times. Significant differences at *P*-value<0.05 level are indicated by different letters

Relationship between NF-κB and Wnt signaling in skin fibroblast cell damage

HG activated inflammatory response including NF-κB and TNF-α signaling. In addition, the NF-κB pathway inhibitor, Bay117082, supply suppressed HG-mediated repression of *FZD8*, Wnt signaling marker gene (10). Therefore, connection between NF-κB and Wnt signaling was further analyzed.

Immunoblotting results indicated that Bay117082 treatment increased the level of β-catenin, slightly higher than Wnt3a (Figures 4A, 4B). In an opposite way, the effect of Wnt3a on IκBα, the most characterized and studied NF-κB regulator, activity was examined. The results of Western blot analysis indicated that Wnt3a treatment did not change both phosphorylated IκBα (p-IκBα) and total IκBα (t-IκBα) levels (Figures 4C, 4D). Taken together, these results suggest that the NF-κB is upstream of β-catenin, the key regulator of Wnt signaling, but Wnt signaling activation via Wnt3a did not change NF-κB activity.

Discussion

Diabetes-induced skin ulcer is hard to be recovered, and is considered as a global health issue in the patients suffering from DM. Skin wound healing process requires coordination of multiple layers of cells including fibroblasts. The fibroblasts are important for synthesizing the ECM and collagen, the structural framework (stroma) for skin tissues. Previous studies on the HG-induced damage of skin fibroblasts identified that abnormal activation of Rac1 and suppression of JNK are tightly associated with skin wound repair (9, 21). Furthermore, transcriptome study regarding HG stress in human skin fibroblast cells identified that many biological processes were altered upon the fibroblasts stressed by HG, including Wnt and inflammatory response

pathways (10). Here, we observed that HG reduces accumulation of the key Wnt signaling regulator β-catenin, but Wnt3a supply recovered HG effects on expressions of β-catenin and its downstream *FZD8* gene (Figures 1B, 1E), suggesting that HG may change β-catenin accumulation to alter Wnt signaling downstream gene expressions. In addition, Wnt signaling activation protected fibroblast cell from HG stress through increased cell proliferation (Figure 2B), implying that Wnt signaling is important for HG-mediated fibroblast damage.

To further understand Wnt signaling activation by Wnt3a, RNA-Seq assay was utilized to analyze. The results determined a large population of differentially expressed genes after Wnt3a stimulation. Among them, 200 genes were down-regulated, while 207 genes were up-regulated. Further, analyses of associated pathways using GO and KEGG database revealed various biological processes and pathways (Table 2 and 3). Compared with the transcriptome data about HG-mediated gene expressions in skin fibroblasts, Wnt3a stimulation changed a largely different group of genes, but Wnt signaling was observed in both analyses (10) (Table 2 and 3). *FZD8* gene expression after HG and Wnt3a stimuli showed that HG represses, while Wnt3a induces *FZD8* gene expression (Figure 2A), indicating that HG and Wnt3a in opposite way regulate Wnt signaling gene.

Furthermore, inhibition of NF-kB pathway through the treatment of Bay117082 induced β-catenin, but Wnt3a supply did not change IκBα activity, the key component of NF-kB signaling, exhibiting the maintenance of total and phosphorylated IκBα levels (Figure 4). These data suggest that at least IKK/NF-kB signaling is the upstream of β-catenin and Wnt3a treatment did not make influence on IκBα activity. In addition, Wnt3a-changed transcriptome did not cover NF-kB and other inflammatory response pathways genes (Table 3). Previously, Bay117082 treatment induced *FZD8* gene expression, but it is reversed by application of Wnt signaling inhibitor, IWR (10). These results are somehow similar with the data presented here, and relationship between Wnt and NF-kB signaling needs to be clarified by testing more markers in the further study. In conclusion, this study identified that HG affects human skin fibroblast cells partially via inhibition of Wnt signaling, and inhibition of NF-kB or activation of Wnt signaling by application of inhibitor or ligand molecules reserve the HG-induced damages. The results presented here will be useful for further understanding molecular basis of diabetes-induced skin ulcer.

Conclusion

Findings of the present study showed the effects of HG stress in skin fibroblast cells. Treatment of Wnt3a to activate Wnt signaling somehow protects HG-induced damages on fibroblasts. Wnt3a treatment induced a large number of gene alterations and inflammatory response signaling located upstream of Wnt signaling to protect HG damages on skin fibroblast cells.

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