**DNA** **methylome and transcriptome alterations in a high-glucose induced cell model and Identification of novel targets of diabetic nephropathy and treatment by Tanshinone IIA**

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#equal contribution

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**Abstract**

Diabetic nephropathy (DN) is a diabetes complication which comes from overactivation of Renin-Angiotensin System, excessive proinflammatory factors, reactive oxygen species (ROS) overproduction, and epigenetic changes. TIIA exhibits protective effects on DN by inhibiting ROS induced by high glucose. A systematic next-generation screening for RNA and genome methylation will provide underling mechanism of DN and targets of TIIA effects. By IPA analysis of 1,780 genes from HG/LG and 1,416 genes from TIIA/HG with significant log2 fold change from RNA-seq, we found inflammation pathways like Leukotriene biosynthesis and Eicosanoid Signaling were activated by HG stimulation while TIIA treatment may enhance glutathione-mediated Detoxification pathway to overcome the resulted excess oxidative stress and inflammation. By correlation analysis of RNA-seq and methyl-seq results, we identified that DNA methylation of a list of DN associated genes, nmu, fgl2, glo, and kcnip2 were altered in HG induced DN model, and TIIA treatment effectively restored the DNA methylation and gene expression. These findings provide novel insights into the understanding of how epigenetic modifications affect the progression of DN and the preventive effect of TIIA.

Key Words: Diabetic nephropathy, Tanshinone IIA, IPA, methyl-seq, RNA-seq

**Introduction**

Diabetic nephropathy (DN) manifested glomerular hyperfiltration and proteinuria in function, and renal hypertrophy, basement membrane thickening, extracellular matrix(ECM) accumulation, glomerulosclerosis, and interstitial fibrosis in histology, and finally developed into renal failure [1]. Pathological factors attributes to development of DN were acknowledged to be a complexation of overactivation of Renin-Angiotensin System, excessive proinflammatory factors, reactive oxygen species (ROS) overproduction, and epigenetic changes[2-4].

Among them, ROS overproduction played an important role in inducing apoptosis and kidney cell damage upon high-glucose (HG) stimulations [5, 6]. Multiple kidney cells were found to generate excessive ROS by stimulation of high glucose[7, 8]. Overexpression of proinflammatory factors, such as transforming growth factor-β1 (TGF-β1), has proved to be highly associated with ECM accumulation and glomerulosclerosis [9]. Overactivation of TGF-β1 would induce excessive ROS, which will in turn enhance the level of TGF-β1 and worsen the condition of DN [10].

Nuclear factor erythroid 2-related factor 2 (Nrf2), one of the most important cellular defense mechanisms with the ability to modulate many phase II detoxifying enzymes by binding to ARE (antioxidant response element) of those genes and maintain cellular redox hemeostasis [11], has shown to be vital in regulating the antioxidative stress response and is essential for the anti-inflammatory response in many clinical and preclinical studies[12]. Accumulating data suggest that many dietary phytochemicals can induce Nrf2-mediated antioxidant/anti-inflammatory signaling pathways[13]. Hence many of them are used for inhibiting DN[14-16].

Tanshinone IIA (TIIA), a diterpene quinone phytochemical isolated from Salvia miltiorrhiza has a long history of application for cardiovascular disease [17]. Notably, TIIA can suppress reactive oxygen species (ROS) and inflammation through activating Nrf2 pathway [18, 19]. Besides cardioprotective effect, TIIA also possesses multiple pharmacological effects, including antioxidant[20], anti- angiogenesis[21], anti-inflammatory[22] and neuroprotective effects [23]., which contributes to its diverse therapeutic spectrum including diabetes[24, 25]. TIIA exhibits protective effects on both acute kidney injury[26, 27] and chronic renal disorders [28, 29].

However, there are very limited evidences of TIIA on DN, which all used rat cells or streptozotocin (STZ) induced type I DN rat model[24, 30, 31]. In addition, the underlying mechanism of action is not clear. A systematic screening for targets of TIIA effects on DN is highly needed.

More and more emerging evidences indicates Epigenetic changes, including DNA methylation, histone post-translational modifications (PTMs), and noncoding RNA-mediated post-transcriptional alterations, are closely related to DN[32-34]. Next-generation sequencing (NGS) on whole genome or epigenome would provide systematic means in analyzing new biomarkers associated with DN which will provide a novel target for treatment. NGS results including RNA-seq and non-coding RNA-seq began to reveal the novel DN associated biomarkers genome wide [35, 36]. However, there lacks whole DNA methylome especially whole methylome and transcriptome synergistically investigations into the pathological changes of DN. This paper will report our work on DNA methyl-seq and mRNA-seq co-alterations using a high-glucose induced mouse kidney mesangial cell model which represents diabetes in vitro. The NGS results comparison between high glucose, low glucose and TIIA will also provide identification of novel targets for diabetic nephropathy and treatment by TIIA.

**Material & Methods**

Materials

Dulbecco's modified Eagle's medium, fetal bovine serum (FBS), penicillin-streptomycin (10,000 U/ml), puromycin, versene, and trypsin-EDTA were supplied by Gibco (Grand Island, NY, USA). TIIA and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Mouse mesangial cell culture

SV40 MES 13 mouse kidney mesangial cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) with 14 mM HEPES (Gibco; Thermo Fisher Scientific, Inc.) and 5% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO2. Mesangial cells were seeded at 1×105 cells/10 cm dish and were treated with serum free medium for 1 day followed by 0.1%DMSO in 30 mM D-glucose (high glucose, HG) or 0.1%DMSO in 5.5 mM D-glucose + 24.5 mM D-mannitol (isotonic control, low glucose, LG), or TIIA (5µM,10 µM, 15 µM dissolved in 0.1%DMSO in low glucose) for 5 days.

Intracellular ROS detection

CM-H2DCFDA (Invitrogen) was used as the probe. Mes-13 cells were treated with 0.1% DMSO in LG, 0.1%DMSO in HG or TIIA (5µM,10 µM, 15 µM in 0.1% DMSO in LG) for 48 h. The cells were grown to 90% confluence, washed with PBS and then harvested using trypsinization, according to the manufacturer’s protocol. The cells were then washed four times and incubated with 10 µM CM-H2DCFDA for 45 min at 37°C in a relatively high humidity (95%) atmosphere containing a controlled level of CO2 (5%) in the dark. Finally, cell-associated mean fluorescent intensity was measured by flow cytometry in FL1 channel excitation and emission wavelengths were 488 and 525 nm, respectively.

Total RNA/DNA extraction, library preparation, RNA-seq and methyl-seq

Total RNA and DNA was extracted from SV40 MES 13 mouse kidney mesangial cells from LG, HG and TIIA groups using the AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA, USA). The quality and quantity of the extracted RNA and DNAsamples were determined with an Agilent 2100 Bioanalyzer and NanoDrop, respectively.A total of 3 RNA and DNA pooled samples from each group were sent to RUCDR for library preparation and sequencing. Briefly, the library of RNA-seq was constructed using the Illumina TruSeq RNA preparation kit (Illumina, San Diego, CA, USA) according to the manufacturer’s manual. Samples were sequenced on the Illumina NextSeq 500 instrument with 50–75 bp paired-end reads, to a minimum depth of 30 million reads per sample. The DNA samples were further processed using an Agilent Mouse SureSelect Methyl-seq Target Enrichment System (Agilent Technologies, Santa Clara, CA) and sequenced on an Illumina NextSeq 500 instrument with 76-bp single-end reads, generating 34–47 million reads per sample.

Data Analysis

Sequencing data quality was checked using *FastQC 0.11.2* software (Andrews). Linux-base bioinformatics software packages *SAMtools* (Sequencing Alignment/Map tools) (Li et al) and *HIDSAT-2* (hierarchical indexing for spliced alignment of transcripts) (Kim et al) were used to sort, deduplicated, index and align reads in RNA sequencing files. DNA methylation data was processed with *Bismark* tool (Kruger and Andrews). All reads were aligned to the mouse reference genome (mm9.2)

*R 3.5.1* (R Core Team) was used for all downstream statistical analysis and visualization of RNA and DNA sequencing data. ADD R PACKAGES!

*RNA-seq data analysis*

*FastQC (version 0.11.2) (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to validate the quality of the raw sequence data. Next, the reads were aligned to the mouse reference genome (mm9.2) using Tophat (version 2.0.9). Reference gene annotations from UCSC were supplied to TopHat (-G genes.gtf); otherwise, default parameters were used. The Cufflinks v2.2.1 [29] program cuffdiff was used to calculate expression levels, using the UCSC gene annotations and default parameters.* *FPKM (Fragments Per Kilobase of Exon Per Million Fragments Mapped) values were calculated using Cufflinks Version 2.0.2 as provided with the Ensembl gene annotation (release 78).*

*Differential Gene Expression Analysis*

Total of 24,421 genes were mapped. Genes with low counts (less than 20 counts in all samples combined) were removed from the analysis. The remaining 13,954 genes were first normalized to fraction per million (FPM) and examined. Two comparisons - high glucose(HG) vs. low glucose (LG), and TIIA in HG vs HG only, were done using an R package *DEGSeq* (Wang et al) to identify differentially expressed genes. The genes with the log2 difference of at least 0.3 and filtered by q-values (as defined by Storey et al, 2003) were selected. The MA plots (log differences vs log means) for the two comparisons are shown in the Figure 7. The RNA expression patterns of the selected genes were further explored to isolate genes that were affected by the HG treatment but restored by the TIIA.

*SureSelect Methyl-seq analysis*

After alignment, DMRfinder (version 0.1) ( software was used to extract methylation counts and cluster CpG sites into DMRs [38]. Each DMR was defined to contain at least three CpG sites. Methylation differences of at least 10% were considered large enough. Genomic annotation was performed with ChIPseeker (version 1.10.3) in R[39].

To examine the associations of DNA methylation and the downstream RNA expression, the differences in percent methylation and RNA expressions for the genes selected in the RNA-seq analysis were plotted against each other. The genes that exhibited DNA hypermethylation in promoter and RNA downregulation, or DNA hypomethylation in promoter and RNA upregulation, were selected as genes of interest for further analysis.

*Ingenuity Pathway Analysis (IPA) Analysis*

Isoforms with log2 ratios greater than 0.3 or less than -0.3, and filtered by q-values were subjected to Ingenuity Pathway Analysis (IPA 4.0, Ingenuity Systems, www.Ingenuity.com). The input isoforms were mapped to IPA's database, and the top related genes, relevant biological functions, diseases and canonical pathways related to HG induced pathological changes and TIIA interventions were identified.

Quantitative polymerase chain reaction (qPCR) validation of genes of interest

qPCR was used to validate the expression trends of selected genes of interest identified by methyl-seq and RNA-seq. First-strand cDNA from isolated 300 ng mRNA from pooled samples was synthesized using TaqMan® Reverse Transcription reagents (Applied Biosystems, Carlsbad, CA, USA).qPCR was carried out using a QuantStudio™ 5 Real-Time PCR System (Applied Biosystems) with SYBR Green PCR Master Mix (Applied Biosystems) with the qPCR primers listed in Table 1.The gene expression fold changes were normalized to the expression of beta-actin using the 2^−ΔΔCT method (RQ values). The gene expressions from HG group were normalized to 1 and the relative fold changes were obtained from the comparation between the other 2 groups to HG group. All the primers were designed and ordered from Integrated DNA Technologies (IDT, Coralville, Iowa, USA).

Statistical analysis

The data are presented as the mean ± standard deviation (std). One-way analysis of variance (ANOVA) test was performed to test for the differences between the mean RQ values of the three treatment groups, followed by a post hoc pairwise comparisons (Dunnett’s test). Differences with p-values less than 0.05 were considered statistically significant.

Results

**TIIA exerted protection effect on intracellular reactive oxygen species (ROS) induced by high glucose**

In mouse kidney mes-13 cells, 2 days treatment of high glucose will induce 1-fold increase of intracellular ROS damage comparing with low glucose group (Figures 1A&E), while co-treatment of TIIA (5µM, 10 µM and 15 µM) could protect mes-13 cells against ROS damage. Excessive ROS is highly associated with apoptosis and kidney cell damage upon high-glucose (HG) stimulations in DN [5, 6] and TIIA treatment has shown a very promising reversal efficacy especially at 5 µM concentration. Hence, in the following NGS study, we treated mes-13 cells at this concentration for 5 days to study the global epigenomics change induced by TIIA in preventing DN.

**Global transcriptome results comparison**

Global gene expressions were ranked in the order of expression log2 fold change. 1,780 genes from HG/LG and 1,416 genes from TIIA/HG with the log2 fold change levels of 0.3 or more (both, positive and negative) were then used as an input to the IPA software. Top 50 annotated genes with the highest log2-fold change in either direction in HG over LG comparison and top 50 annotated genes with the highest log2-fold change in either direction in TIIA over HG were listed in Table 2 and 3 respectively. Doughnut Heatmap (Figure 2A) demonstrates the 213 overlapping genes with log2 fold changes greater than 0.3 and filtered by q-values which show reversal of the effect of HG treatment by TIIA. As indicated in the Venn diagrams, there are 387 genes increased in HG versus LG and 1,517 genes decreased in TIIA versus HG. Among them, same 124 genes both increased in HG over LG and decreased in TIIA over HG (Figure 2B). There are 296 genes decreased in HG over LG and 1,209 genes increased in TIIA over HG. Among them, same 89 genes both decreased in HG over LG and increased in TIIA over HG (Figure 2C). Those 124 overlapping genes from HG/LG and 89 from TIIA/HG which show the opposite trends in the comparisons were marked as candidates for the genes of interest.

**SureSelect Methyl-seq analysis**

To understand the involvement of DNA methylation in DN, we determined the single-base-resolution DNA methylation of mouse kidney mesangial cells from LG, HG and TA groups.A comparison of the methylation landscape across the treatments showed that overall methylation levels differed by the region but not by treatment, e.g. methylation ratios were much lower in the promoter regions compared to body and intergenic (downstream) regions (Figure 3a). Majority of DMRs consisted of a small number of CpGs (3 to 7) (Figure 3b), with nearly a quarter of CpG located in the promoter region (Figure 3c).

**Correlation of SureSelect Methyl-seq results with RNA-seq results**

Mounting evidences have suggested that the methylation status alteration of gene promoters, unlike other regions, caused reversed gene expression change: hypermethylation of coding or noncoding gene promoters correlates with the reduced expression of them and hypomethylation correlates with increased expression[40]. Based on this notion, we prepared quadrantal graphs to show the association between DNA methylation and gene expression of the 213 overlapping genes from the RNA-seq results (Figures 4a and b). The genes with green dots (corresponding to promoters) in the upper left and the lower right quadrants suggested reversed alteration of methylation in promoters with RNA expression levels. The list of the genes is presented in Table XX (MAKE IT!). DNA methylation level differences of these genes along with the gene expression differences are presented as Lollipop plots (Figure 6).

**Validation of selected gene expression which shows close correlation between RNA-seq and methyl-seq by quantitative real‑time RT‑PCR**

Genes of interest expression in HG were normalized to 1. In Figure 5, relative expression of gulo and kcnip were significantly decreased from 1.65 to 1 and 1.29 to 1 in comparing LG with HG and increased from 1 to 1.54 and 1 to 1.20 from HG to TIIA respectively (p<0.05). Relative expression of fgl2 were significantly increased from 0.67 to 1 from LG group to HG group and decreased from 1 to 0.74 from HG to TIIA (p<0.05). The relative expression of nmu were increased from 0.80 to 1 (from LG to HG) and decreased from 1 to 0.84 (from HG to TIIA). All these change trend correlates well with the RNA-seq results (Table 4).

**Lollipop figures show the association between SureSelect methyl-seq and RNA-seq results**

The Lollipop plots (Figures 6A, B, C and D) provides in-depth understanding of RNA expression and DNA methylation difference within the HG/LG and TIIA/HG comparisons. The length of the stem corresponds to the methylation ratio, and the color of the bubble codes for the different methylation regions (exon: blue; intron: white; promoter: red; distal intergenic region: purple). RNA expression of genes of interest are also listed in the figure. The Lollipops figures are in good accordance with SureSelect methyl-seq results and demonstrate the association between DNA promoter methylation ratio and RNA expression. Fgl2 and NMU indicate a methylation ratio decrease in promoter region in HG/LG and the ratio increase in TIIA/HG. In addition, the genes expression from RNA-seq shows an increase in HG/LG and decrease in TIIA/HG. Gulo and Kcnip2 have opposite change in DNA promoter methylation ratio and gene expression with Fgl2 and NMU. These results suggest treatment of TIIA can reverse HG influence in the 4 genes of interest.

**Discussion and Conclusion**

**Top differentially canonical pathways, disease and Tox influenced by HG and treatment by TIIA identified by IPA analysis**

Figure 8 indicates the 10 most significant associated canonical pathways identified by IPA from all significant and reliable Differentially expressed genes in HG versus LG (Figure 8a) and TIIA versus HG groups (Figure 8b) from mes-13 cells after 5 days treatment. In the top 2 significant associated pathways in the comparison group of HG versus LG, HG can induce both Leukotriene biosynthesis and Eicosanoid Signaling, which are both highly related to enhance proinflammation factors like leukotrienes, prostaglandin, cyclooxygenases (COX-1 and COX-2), promote inflammation and amplify immune response. Leukotrienes are proinflammatory metabolites of arachidonic acid(AA) that activate and amplify innate and adaptive immune responses[41]. They can induce leukocyte aggregration, activate phagocyte and generate proinflammatory factors[42]. Four major types of eicosanoids, prostaglandins, lipoxins, leukotrienes and thromboxanes are generated by AA through Prostaglandin endoperoxide synthases or lipoxygenases[43]. Eicosanoids can modulate complicated oxidative response, inflammation, allergy and carcinogenesis[44]. Our in vitro long-term HG treatment seems to be able to enhance oxidative stress and inflammation response in mouse kidney mesangial cells mainly via Leukotriene biosynthesis and Eicosanoid Signaling pathway.

In the top 2 significant associated pathways in the comparison group of TIIA versus HG, TIIA can influence Liver X receptor (LXR)/ The retinoid X receptors (RXR) activation and enhance glutathione-mediated Detoxification. LXR/ RXR has a close relation with the regulation of metabolism of glucose, lipid and cholesterol and inflammation [45]. Tripeptide glutathione (GSH) forms thioether conjugates with leukotrienes, prostaglandin and other chemicals, which can be subsequently degraded by Gamma-glutamyl hydrolase or γ-glutamyl transpeptidase, and dipeptidases[46]. Our findings suggest TIIA treatment can restore the cellular response induced by HG mainly targeting the above two pathways.

The Tox Analysis by IPA is to indicate most associated biological processes and toxicological responses to xenobiotic influence. In the top 10 mostly associated tox changes (Figure 9), majority toxicological response in HG/LG and TIIA/HG are both mainly associated with kidney disorders, which suggest the suitability of High glucose induced mes-13 cell model as an in vitro DN cell model.

**Correlated genes of interest**

Based on the analysis of sureselect-methy-seq and RNA-seq results, we identified 4 most relevant genes , in which HG can induce DN pathological associated changes in gene expression and accompanying with an opposite DNA methylation change in DNA promoter, while TIIA can restore the alteration to normal.

NMU, a neuropeptide belongs to the neuromedin family can generate active neuropeptides and regulate pain, stress, cancer and inflammatory diseases [47]. Recent findings indicate that NMU can act directly on pancreas β cells through NMUR1 in an autocrine or paracrine fashion to suppress insulin secretion[48]. In our in vitro system, HG can induce a very according high NMU expression fold change (17.495) over LG, which is the highest fold change in HG/LG comparison (Table 4) accompanying with a decrease in DNA methylation (-0.526) of NMU promoter, which suggest increase of NMU by HG correlates with the decrease of DNA methylation in its promoter region. TIIA can reverse the change in gene expression and DNA methylation and indicating the potential therapeutic target on NMU. Fibrinogen-like protein 2 (Fgl2) is a novel prothrombinase. Increased fgl2 level was found to be highly correlated with the circulating TNF-α levels and severity of mouse type 2 diabetic nephropathy[49]. Like NMU, HG can induce a very according high fgl2 expression fold change (5.346) over LG accompanying with a decrease in DNA methylation (-10.324) of NMU promoter. TIIA treatment also demonstrate a relative restoration effect on both gene expression and DNA methylation.

L-gulono-gamma-lactone oxidase (GLO), a necessary enzyme for ascorbic acid synthesis, was found to be decreased in diabetic rats [50]. In a Type 2 rat diabetes model, Potassium voltage-gated channel interacting protein 2 (Kcnip2/KChIP2) were found to be down-regulated[51]. Our results (Table 4) echoes the above findings that HG can decrease GLO and Kcnip2 greatly(both 0.121) and correlates with an increase in the methylation ratio in their promoters (18.530 and 11.567 respectively). TIIA can effectively reverse the alteration in both gene expression and DNA methylation.

Those four genes of interest will be targets for our further investigation.

In conclusion, this study demonstrated the TIIA protective effect of against HG induced damage to kidney. Using SureSelect Methyl-seq and RNA-seq, we provided a quantitative global profile of the methylome and transcriptome in mouse kidney mesangial cells from LG and HG with or without TIIA treatment. IPA analysis identified inflammation pathways like Leukotriene biosynthesis and Eicosanoid Signaling were activated by HG stimulation while TIIA treatment may enhance glutathione-mediated Detoxification pathway to overcome the resulted excess oxidative stress and inflammation. Importantly, we identified that DNA methylation of a list of DN associated genes, nmu, fgl2, glo, and kcnip2 were altered in HG induced DN model, and TIIA treatment effectively restored the DNA methylation and gene expression. These findings provide novel insights into the understanding of how epigenetic modifications affect the progression of DN and the preventive effect of TIIA.

Notes

The authors declare that there are no conflicts of interest.

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Table and Figure Legends

Tables

Table 1. Real-time q-PCR Primers information of validated genes of interest

Table 2. Top 50 annotated genes showing the highest log2-fold change in either direction in high glucose treated group (HG) over low glucose treated group (LG), ranked by log2-fold change

Table 3. Top 50 annotated genes showing the highest log2-fold change in either direction in 5µM TIIA treated group (TIIA) over HG, ranked by log2-fold change

Table 4 Correlation of DNA promoter methylation ratio from methyl-seq and fold change of gene expression from RNA-seq for genes of interest

Figures

Figure 1. Effects of TIIA on production of intracellular reactive oxygen species (ROS) induced by 2 day treatment of high glucose (HG) In mouse kidney mes-13 cells via flow cytometry. Two days treatment of HG induced increase of intracellular ROS damage comparing with low glucose (LG) group(A), co-treatment of 5µM TIIA (B), 10 µM TIIA (C) and 15 µM TIIA(D) could protect mes-13 cells against ROS damage from HG. Relative ROS fold change normalized by LG (E) are expressed as means ± Std for 3 independent replicates and significant (p<0.05, \*; p<0.01, \*\*) difference comparing with HG are indicated.

Figure 2. Overview of the differentially expressed genes in 2 comparisons among 3 groups (HG/LG and TIIA/HG). (A) Doughnut Heat map of top ? Genes with differential expression that appeared in HG versus LG group and TIIA versus HG group. (B, C) Venn diagrams comparing the up-regulated (red) and down-regulated genes (green) between HG versus LG group and TIIA versus HG group. Genes with log2 fold changes greater than 0.3 were counted.

Figure 3. Sureselect Methyl-seq results overview

Figure 4 Quadrantal graph shows correlation between RNA-seq and methyl-seq. The red circle indicates those genes with reversed change of methylation and RNA expression.

Figure 5. RNA qPCR validation for the genes of interest. The gene expressions from HG group were normalized to 1 and the relative fold changes were obtained from the comparation between the other 2 groups to HG group. All the data are presented are expressed as means ± Std for 3 independent replicates and significant (\*, p<0.05) difference comparing with HG are indicated

Figure 6. In-depth analysis of DNA methylation of nmu(A), fgl2(B), gulo(C) and kcnip(D) in looipops figures

Figure 7. DESeq2 FPM normalization, log2 differences of at least 0.3 (1.23 fold-change) P-values from DEGseq between HG/LG (A) and TIIA/HG (B)

Fig. 8 Canonical pathways identified by IPA for all significant and reliable Differentially expressed genes in HG versus LG (a) and TIIA versus HG (b) from mes-13 cells after 5 days treatment. Canonical pathways are displayed as the –log(p-value) (upper) with the threshold of 1.5 indicating the minimum significance level. Length of the bars represents the significant associations. The ratio indicates the number of Differentially expressed genes in the comparison that map to a given canonical pathway divided by the total number of genes in that pathway.

Figure 9. The 10 most associated tox results related to HG versus LG (upper) and TIIA versus HG(lower)

Table 1. Real-time q-PCR Primers information of validated genes of interest

|  |  |  |
| --- | --- | --- |
| Genes | Primer Sequence (5’-3’) | Amplicon Size (bp) |
| Bmp8b | F: GTGCATCTACCCACTGAACTC  R: AGCAGAGAAATGGCACTCAG | 93 |
| Fgl2 | F: AAGTGTTCCAAGTGTCCCAG  R: TGCTGTTTCTGTGATCAGGG | 101 |
| Gulo | F: AAACTGGGCGAAGACCTATG  R: GATGTCTGAAGGCGAGTGG | 105 |
| Kcnip2 | F: GAGAGTTTGTCCGAATCCCG  R: TCTCTGCGTGTGAACTTGG | 106 |
| β-actin | F: ACCTTCTACAATGAGCTGCG  R: CTGGATGGCTACGTACATGG | 106 |

Table 2 Top 50 annotated genes showing the highest log2-fold change in either direction in high glucose treated group (HG) over low glucose treated group (LG), ranked by log2-fold change

|  |  |  |  |
| --- | --- | --- | --- |
| Increased (HG/LG) | | Decreased (HG/LG) | |
| Symbol | Log2 fold change | Symbol | Log2 fold change |
| NMU | 4.129 | ZIC2 | -4.626 |
| THEM5 | 3.959 | Gm14827 | -3.848 |
| DHH | 3.766 | LYZ | -3.848 |
| HSD3B1 | 3.659 | NUTM1 | -3.626 |
| CD300A | 3.544 | Sh3bgr | -3.626 |
| Cyp2ab1 | 3.544 | GRIN1 | -3.501 |
| FBXL13 | 3.544 | Gulo | -3.041 |
| INSYN2 | 3.544 | KCNIP2 | -3.041 |
| ARHGAP6 | 3.281 | TSSK2 | -3.041 |
| C130021I20Rik | 3.281 | C11orf98 | -2.848 |
| C4A/C4B | 3.281 | HIST2H2BF | -2.848 |
| DPEP2 | 3.281 | IL23R | -2.848 |
| E130102H24Rik | 3.281 | TNFRSF25 | -2.848 |
| ENTPD1 | 3.281 | SMYD1 | -2.742 |
| PBLD | 3.281 | ANKRD61 | -2.626 |
| Pga5 | 3.281 | Aox4 | -2.626 |
| SCN1A | 3.281 | C11orf65 | -2.626 |
| Snora5c | 3.281 | EPHX4 | -2.626 |
| Zfp345 (includes others) | 3.281 | FCER1G | -2.626 |
| KCNJ15 | 3.129 | Mir1191 | -2.626 |
| OBSCN | 3.129 | Mir8091 | -2.626 |
| Cd59a | 2.959 | NPAS3 | -2.626 |
| HNF4A | 2.959 | MAMDC2 | -2.501 |
| ACSM2A | 2.766 | MYO7B | -2.501 |
| Ces2f | 2.766 | Olfr99 | -2.501 |
| HPGDS | 2.766 | C19orf66 | -2.363 |
| ISLR2 | 2.766 | GNG8 | -2.363 |
| KLK3 | 2.766 | MESP2 | -2.363 |
| LDHD | 2.766 | SLC4A5 | -2.363 |
| LIPN | 2.766 | Bmp8b | -2.157 |
| Mkln1os | 2.766 | C1QTNF3 | -2.157 |
| NCKAP5 | 2.766 | ATP2A1 | -2.041 |
| KIAA1324 | 2.681 | CD160 | -2.041 |
| AKAP5 | 2.544 | Ces1f | -2.041 |
| CFAP45 | 2.544 | CYP4F12 | -2.041 |
| Chn1os3 | 2.544 | DUSP13 | -2.041 |
| Cyp2j5 | 2.544 | EPSTI1 | -2.041 |
| CYP4F22 | 2.544 | Gbp8 | -2.041 |
| FAM19A5 | 2.544 | GJA4 | -2.041 |
| GPR132 | 2.544 | MAPK10 | -2.041 |
| ICAM1 | 2.544 | MC1R | -2.041 |
| PKNOX2 | 2.544 | MYO16 | -2.041 |
| RAB39B | 2.544 | Nrp | -2.041 |
| SLC22A6 | 2.544 | Serpinb9f (includes others) | -2.041 |
| SOX21 | 2.544 | SLC23A1 | -2.041 |
| TCP11 | 2.544 | TMEM266 | -2.041 |
| FGL2 | 2.418 | TMOD1 | -2.041 |
| Gm19589 | 2.418 | Wfdc3 | -2.041 |
| 4930447K03Rik | 2.281 | CCDC116 | -1.967 |

Table 3 Top 50 annotated genes showing the highest log2-fold change in either direction in 5µM TIIA treated group (TIIA) over HG, ranked by log2-fold change

|  |  |  |  |
| --- | --- | --- | --- |
| Increased (TIIA/HG) | | Decreased (TIIA/HG) | |
| Symbol | Log2 fold change | Symbol | Log2 fold change |
| GSTA5 | 4.727 | LCN2 | -4.756 |
| Gsta1 | 4.523 | ACE2 | -4.586 |
| GSTA1 | 3.999 | Gm19589 | -4.46 |
| Sh3bgr | 3.999 | HSPA12A | -4.46 |
| UGT2B28 | 3.999 | Iigp1 | -4.46 |
| IL23R | 3.906 | STEAP4 | -4.393 |
| HTRA3 | 3.806 | CCDC33 | -4.323 |
| KCNN4 | 3.806 | ABCA12 | -4.171 |
| ADAM32 | 3.584 | LPL | -4.001 |
| Snora2b | 3.584 | THEM5 | -4.001 |
| Ly6a (includes others) | 3.321 | Trim30a/Trim30d | -3.908 |
| LYZ | 3.321 | MS4A10 | -3.808 |
| MSC | 3.321 | Dpt | -3.701 |
| NOSTRIN | 3.321 | S100G | -3.701 |
| NYX | 3.321 | CD300A | -3.586 |
| TNFRSF25 | 2.806 | CYP4F22 | -3.586 |
| Bmp8b | 2.584 | IRF4 | -3.586 |
| FCER1G | 2.584 | Ly6a (includes others) | -3.586 |
| Itgb2l | 2.584 | NR1I3 | -3.586 |
| Nkx6-3 | 2.584 | TLL1 | -3.586 |
| WSCD2 | 2.584 | UBE2QL1 | -3.586 |
| Dusp13 | 2.458 | NAD+ | -3.481 |
| Rorc | 2.458 | CCDC160 | -3.46 |
| Gm4432 | 2.414 | GLI2 | -3.323 |
| RAPSN | 2.368 | mir-761 | -3.323 |
| CHRM1 | 2.321 | OBSCN | -3.171 |
| Dpf3 | 2.321 | PLA2R1 | -3.171 |
| GJA4 | 2.321 | RCSD1 | -3.171 |
| MESP2 | 2.321 | 3830432H09Rik | -3.001 |
| Mir1191 | 2.321 | A630001G21Rik | -3.001 |
| MPZ | 2.321 | Snord19 | -3.001 |
| NALCN | 2.321 | INMT | -2.971 |
| SLC23A1 | 2.321 | CCL5 | -2.808 |
| SLC4A5 | 2.321 | HIST1H2BI | -2.808 |
| Snora43 | 2.321 | PHF24 | -2.808 |
| TSSK2 | 2.321 | AGT | -2.645 |
| Wfdc3 | 2.321 | FERMT1 | -2.586 |
| Snora23 | 2.169 | GALNT18 | -2.586 |
| TFR2 | 2.169 | GPR132 | -2.586 |
| ANKRD61 | 2.114 | IL23A | -2.586 |
| ANKRD63 | 1.999 | PDE11A | -2.586 |
| ARSJ | 1.999 | PLXNC1 | -2.586 |
| ATP2A1 | 1.999 | PRDM1 | -2.586 |
| C11orf98 | 1.999 | RBP4 | -2.586 |
| C19orf66 | 1.999 | Timd2 | -2.586 |
| EPHX4 | 1.999 | TTYH1 | -2.586 |
| GNG8 | 1.999 | TULP2 | -2.586 |
| GRIN1 | 1.999 | HIST1H2AL | -2.504 |
| Gulo | 1.999 | RSAD2 | -2.475 |
| Kcnip2 | 1.999 | KIAA1324 | -2.46 |

Table 4 Correlation of DNA promoter methylation ratio from SureSelect Methyl-seq and fold change of gene expression from RNA-seq for genes of interest

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Genes of interest | DNA Promoter methylation ratio of HG/LG | DNA Promoter methylation ratio of TIIA/HG | Fold change of expression in HG/LG from RNA-seq | Fold change of expression in TIIA/HG from RNA-seq |
| Fgl2 | -10.324 | 7.265 | 5.346 | 0.273 |
| Gulo/(GLO) | 18.530 | -14.526 | 0.121 | 3.997 |
| Kcnip2/KChIP2 | 11.567 | -4.748 | 0.121 | 3.997 |
| Nmu | -0.526 | 12.378 | 17.495 | 0.555 |

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