Activation of the JAK1 / STAT1 Signaling Pathway is Associated with Prdx6 Expression Levels in Human Epididymis Epithelial Cells

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Research Article

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Abstract

I. Background: Peroxiredoxin 6 (Prdx6) is widely expressed in mammalian tissues. Our previous study demonstrated that Prdx6 was expressed in human epididymis and spermatozoa, and the protective role of Prdx6 in human spermatozoa was also reported. In this study, we demonstrate the potential role and mechanism of Prdx6 in human epididymis epithelial cells (HEECs).

II. Methods and Results: Western blotting was used to measure expression levels of key proteins in the JAK / STAT signaling pathway. Digital gene expression analysis (DGE) was used to identify gene expression patterns in control HECs and in HECs after Prdx6-RNA interference (P6-RNAi). The DGE analysis identified 589 up-regulated and 314 down-regulated genes (including Prdx6) in Prdx6-RNAi (P6-RNAi) HEECs. Thirteen significantly different pathways were identified between the two groups, with the majority different expressed genes belonging to the CCL, CXCL, IL, and IFIT families. In particular, the expression levels of IL6, IL6ST, and eighteen IFN related genes were significantly increased in the condition of the down-regulated expression of Prdx6. Compared to control HEECs, the expression levels of JAK1, STAT1, phosphorylated JAK1 and STAT1 were significantly increased, while the expression levels of SOCS3 was significantly decreased in P6-RNAi HEECs. The Malondialdehyde (MDA) level and total antioxidant capacity in P6-RNAi HEECs were significantly increased and decreased compared to that of control, respectively.

III. Conclusions: We speculated that knockdown of Prdx6 resulted in higher levels of ROS in HEECs, which in turn, activated the JAK1 / STAT1 signaling pathway induced by IL-6 receptor and IFN.

Background

Peroxiredoxins (Prdxs) are antioxidant enzymes and are composed of six family members (Prdx1-6)[1]. Prdx1-5 contains 2-Cys, while Prdx6 contains only 1-Cys[2]. Prdx6 is an abundant cellular protein with peroxidase, phospholipase A2 (PLA2), and lysophospholipid acyl transferase (LPCAT) activity[3].

The primary function of Prdxs is to prevent oxidative damage induced by reactive oxygen species (ROS) [4]. The relationship between Prdxs, inflammation, and immunity has also been widely reported. Prdx1 act as an immunomodulator in macrophages and endothelial cells[5], and has been associated with the occurrence of atherosclerosis and rheumatoid arthritis[6]. Moon et al. (2006) demonstrated the inhibitory function of Prdx2 in immune cell responsiveness[7], while Szabó-Taylor et al. (2012) demonstrated the role of Prdx2 in chronic inflammation and the relationship between Prdx2 and the occurrence of rheumatoid arthritis[8]. Prdx3 is the only member that lacks an immunomodulatory function, and its deficiency has been shown to increase abnormal lipid accumulation in adipose tissue[9]. Prdx4 inhibits inflammation induced by oxidative stress in various tissues[10], and its molecular mechanism may be via the regulation of NF-κB, which is an important pro-inflammatory transcription factor[11]. Prdx4 expression has also been shown to be associated with rheumatoid arthritis[12]. Prdx5 regulates inflammatory processes through the Trx system[13], while Prdx6 has been shown to be involved in
regulating cell proliferation, apoptosis, embryonic development, lipid metabolism, immune response, and osteogenic differentiation[14-21]. Furthermore, Prdx6 has been shown to activate the NF-κB / AP-1 and JAK pathways necessary for the development of rheumatoid arthritis[22]. In brief, the Prdxs family, especially Prdx6, is associated with immunity.

Multiple developmental and immunological processes are regulated by the JAK / STAT signaling pathway[23]. There are four JAKs and seven STATs found in mammals. The JAK / STAT signaling pathway is triggered by the binding of extracellular ligands to cell surface receptors to activate receptor-associated JAKs; JAKs phosphorylate and activate cytoplasmic STAT dimers; phosphorylated STAT translocate to the nucleus and regulates target gene expression[24]. The suppressor of cytokine signaling (SOCS) proteins provides selective negative feedback to STAT activation to prevent over-stimulation of the immune system. Of the eight members, only SOCS1 and SOCS3 have been well studied. SOCS1 and SOCS3 have been shown to inhibit components of the JAK/STAT and other cell signaling pathways in a highly cell-type-specific manner[25-27].

Mammalian epididymis provides the critical environment for spermatozoa to develop motility and fertilization ability[28]. Our previous study showed that Prdx6 was expressed in the human epididymis[29], while Fernandez et al (2018) demonstrated that Prdx6 functioned in antioxidant defense in human spermatozoa[30]. In the current study, based on the role of Prdx6 in the immune response and the regulatory role of the JAK / STAT signaling pathway during the immune response, we aimed to discuss whether there was relationship between Prdx6 and the JAK / STAT signaling pathway in the human epididymis epithelial cells (HEECs), and which member of JAK / STAT families participated in the process.

Materials And Methods

Culturing HEECs

HEECs[31] were kindly provided by Daniel G. Cyr (INRS-Institut Armand Frappier, University of Quebec, Laval, Quebec, Canada). HEECs were cultured in DMEM/HAM F12 media with penicillin (50 U/ml), streptomycin (50 lg/ml), L-glutamine (2 mM), insulin (10 lg/ml), transferrin (10 lg/ml of), hydrocortisone (80 ng/ml), testosterone (5 nM), epidermal growth factor (10 ng/ml), cAMP (10 ng/ml), sodium selenium (2 ng/ml), tocopherol (200 ng/ml), retinol (200 ng/ml), and 10% fetal bovine serum [FBS] [Sigma-Aldrich]. Cells were seeded in culture plates coated with collagen IV (BD Biosciences, Mississauga, Canada) and incubated in a humidified chamber at 32°C with 5% CO₂.

Construction of the Prdx6 shRNA expression plasmid

Short hairpin RNA (shRNA) sequences targeting the mRNA of the human Prdx6 gene (Gens ID: 9588) were designed using the online tool (http://www.genesil.com/siRNA design.asp)[32] (Table 1). The Prdx6 shRNA sequences were then inserted into the endonuclease loci of BamHⅠ and Hind III in the pGenesil-1
vector (Wuhan GeneSil Biotechnology, Wuhan, China). The shRNA sequences were specific and did not target mRNA sequences of other known human genes (Table 5).

**Plasmid Transfection**

HEECs at 80% confluence were transfected with the Prdx6 shRNA expressing construct using the FuGENE HD Transfection Reagent (Roche, Mannheim, Germany). In addition, HEECs were transfected with non-targeting shRNA constructs to be used as the control. Transfections were performed in triplicate.

**Protein extractions and Western blot analysis**

Total proteins were extracted from 48hr post-transfected HEECs using RIPA lysis buffer (P0013B, Beyotime Biotechnology, China) supplemented with PMSF (ST506, Beyotime Biotechnology) and protease phosphatase inhibitors (P1050, Beyotime Biotechnology). Total proteins were then separated on a 10% (w/v) SDS-PAGE for JAK1 and STAT1 detection and 12% (w/v) SDS-PAGE for Prdx6, SOCS3 and GAPDH detection. After electrophoresis, transferred and blocked membrane, the membrane was incubated with primary antibodies overnight at 4°C. Anti-JAK1 antibody (ab133666, Abcam, UK) was used at 1:800; anti-JAK1 (phospho Y1022 + Y1023) (ab138005, Abcam) at 1:500; anti-STAT1 (ab47425, Abcam) at 1:500; anti-STAT1 (phospho Y701) (ab29045, Abcam) at 1:500; anti-Prdx6 (ab92322, Abcam) at 1:1000; anti-SOCS3 (ab16030, Abcam) at 1:1000; and anti-GAPDH (ab181602, Abcam) at 1:2000. After incubation, the membranes were washed with PBS-Tween (1000:1) and then incubated with the relevant HRP-labeled secondary antibody for 2h at room temperature. X-ray film (Bio-Rad, Hercules, CA) was used for chemiluminescent detection of target proteins. Experiments were performed in biological triplicates.

**Detection of MDA levels and total antioxidant capacity of HEECs**

The MDA levels and total antioxidant capacity in HEECs of control and Prdx6-RNAi were detected with Commercial kits (S0131S, Beyotime Biotechnology; A015-2-1, Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocol.

**Sample preparation and RNA isolation**

Total RNA was extracted from 48 hr post-transfected cells using TRIZOL (Invitrogen, Carlsbad, CA, USA). RNA quality was measured using ultraviolet spectrophotometry and denaturing agarose gel electrophoresis.

**DGE library preparation and sequencing**
Library preparation and sequencing were performed by BGI using the Illumina Gene Expression Sample Prep Kit and Solexa Sequencing Chip (flowcell) on the Illumina Cluster Station and Illumina HiSeq™ 2000 System.

Data transformation and gene annotation

Raw solexa sequences were transformed using the following steps: removal of the 3’ adaptor sequence, empty reads, and low-quality tags; selection of 21nt read length tags; removal of single copy tags; generation of Clean Tags. After quality assessment, the clean tags were used to generate alignment statistics between the P6-RNAi and control cells.

All clean tags were mapped to the reference sequence and only 1bp mismatch was considered during alignment. The number of unambiguous clean tags for each gene was calculated and then normalized to TPM (number of transcripts per million clean tags)[33-34].

Detection of differential gene expression

Differentially expressed genes between the two groups of transfected HEECs were determined using the Audic-Claverie method (1997)[35]. The threshold used to determine significant differences in gene expression was based on False Discovery Rate (FDR) ≤0.001 and the absolute value of log\(_2\)Ratio≥1, as described in Benjamini, Yekutieli (2001)[36].

Gene ontology functional enrichment analysis for DEGs

Gene Ontology (GO) functional enrichment and Genomes (KEGG) pathway enrichment analysis were performed to uncover biological function and metabolic pathways of the differentially expressed genes, respectively. GO analysis was performed based on the methods used by Ye et al. (2006)[37]. Significantly enriched metabolic and signal transduction pathways were identified using the KEGG public database[38].

Pathway enrichment analysis for DEGs

Pathway enrichment analysis was performed similarly to that of GO analysis.

Real-time PCR

Total RNA was extracted using TRIZOL (Invitrogen, Carlsbad, CA, USA) based on the manufacturer's instructions. The first strand of cDNA was generated with ReverTra Ace (MMLV Reverse Transcriptase RNase H–) (TRT-101, Toyobo Co., Ltd., Osaka, Japan). Real-time PCR was performed with EvaGreen 2×
qPCR Master Mix (Applied Biological Materials, Inc., Vancouver, Canada) with Rotor-Gene Q (QIAGEN, Hilden, Germany). The results were expressed as the real-time quantity of the target gene / GAPDH. Gene-specific primer sequences and PCR conditions are shown in Table 6.

**Results**

**The expression levels of PRDX6 protein and Prdx6 mRNA**

The expressions of PRDX6 protein and Prdx6 mRNA were both significantly decreased in the Prdx6-RNAi (P6-RNAi) HEECs compared to the control cells (Figure 1A - C). This confirmed that our shRNA sequence targeting Prdx6 was effective.

**Sequencing Quality**

The distribution patterns of total and distinct tags were similar between HEECs transfected with P6-RNAi and non-targeting shRNA construct. This suggested the absence of bias in the construction of the two libraries (Figure 2). Tags with copy numbers > 100 were dominant for both P6-RNAi and control HEECs, while the majority of distinct tags had copy numbers < 5 (Figure 2). This indicated that low mRNA levels were more significant.

**Differential Gene Expression**

There were 903 differentially expressed genes between the control and P6-RNAi transfected HEECs. Compared to the control cell, 589 genes were up-regulated and 314 were down-regulated (including Prdx6) in P6-RNAi transfected cells (Figure 1D).

**Real-time PCR**

Ten up-regulated and ten down-regulated genes were randomly selected for real-time PCR validation. Consistency was observed between real-time PCR and DGE results (Figure 3).

**Functional annotation of DEGs**

DEGs were categorized into biological processes, molecular function, and cellular components (Figure 4). The biological processes contained cellular protein metabolic, metabolic, multi-organism, response to other organism, and response to biotic stimulus. The molecular function was cytokine activity. The cellular components involved in ribosomal subunit, ribosome, cytoplasmic part, cytoplasm, organelle part, intracellular organelle part, membrane-bounded organelle, intracellular membrane-bounded organelle, organelle, intracellular organelle, intracellular part, and intracellular.
Pathway analysis of DEGs

There were 13 significantly different pathways between P6-RNAi and control HEECs (Table 1). The total number of related genes in the significantly different pathways were 80, of which, 44 genes belonged to the CCL, CXCL, IL, and IFIT family. The four gene families are reported to be associated with immunity.

The mRNA expression levels of JAKs, STATs, and SOCSs

Compared to control cells, the expression levels of JAK1 and STAT1 were significantly increased, while the expression level of SOCS3 was significantly reduced in P6-RNAi HEECs (Table 2).

The expressions of proteins in JAKs / STAT signaling pathway

We measured the expression levels of JAKs, STATs, and SOCSs in HEECs. Compared to control HEECs, expression levels of JAK1 and STAT1 were significantly increased in P6-RNAi HEECs. In addition, the expression level of SOCS3, which is an inhibitor of the JAK / STAT signaling pathway, was significantly reduced in P6-RNAi HEECs (Figure 5A-B).

Activation of the JAK1 / STAT1 signaling pathway by the IL-6 receptor family and IFNγ in P6-RNAi HEECs

Compared to control cells, the expression levels of IL-6, IL6ST, and downstream genes (i.e., PIM-3, and cytokines) were significantly increased in P6-RNAi HEECs (Table 3).

Although differences of expression levels in IFNγ and IFNγ receptor were not observed between the control and P6-RNAi HEECs, the majority of IFNγ related genes (IFRD1, IRF9, IFI6, IFI27, MX1, IFI44, IFI44L, IFIT1, IFIT3, IFITM1, IFIT2, IFIH1, IFIT5, IFI30, IFITM3, GBP1, IFI16, IRF7) showed significantly higher expression (Table 4). Furthermore, the expression levels of downstream genes of IFNγ signaling (OAS1, OAS2, OAS3, OASL, HLA-A) were significantly higher in P6-RNAi HEECs (Table 4).

The Malondialdehyde (MDA) levels and total antioxidant capacity of HEECs

The MDA level in Prdx6-RNAi HEECs was significantly increased compared to that of control. The total antioxidant capacity, meanwhile, showed the opposite trend (Figure 6).

Discussion
Our previous study demonstrated that Prdx6 was expressed in human epididymis, seminal fluid, and spermatozoa[29], and the protective role of Prdx6 in human spermatozoa was also reported[28]. Here, we try to investigate the potential role of Prdx6 in HEECs.

The DEG results showed that the differentially expressed genes between P6-RNAi and control HEECs mostly belonged to the CCL, CXCL, IL, and IFIT families that are associated with immunity. On the other hand, it is well known that multiple developmental and immunological processes were regulated by the JAK / STAT signaling pathway[39]. This prompted us to investigate whether down-regulation of Prdx6 could activate the JAK/STAT signaling pathway. Interestingly, as we had speculated, we were able to detect the up-regulated expressions of JAK1 and STAT1, and the significantly decreased expression of SOCS3 in P6-RNAi HEECs. The same expression trend of JAK1, STAT1, and SOCS3 also be found in the DEG analysis.

To further verify the activation of JAK1/STAT1 signaling pathway, we analyzed the upstream and downstream genes of the pathway. The JAK-STAT pathway has been widely reported to be activated by IL-6 and IFNγ. IL-6 is required for the activation of the JAK-STAT pathway during myocardial infarction[40], in myelomas[41], head and neck tumors[42], and primary breast cancers[43]. IFNγ is one of the most important cytokines and plays an important role in defense against microbial infections by producing various cytokines and inducing autophagy[44]. IFNγ activation of the JAK2 / STAT1 signaling pathway has been reported in EBV (+) gastric cancers[45], though the activation of STAT1 in primary mammary carcinomas was found to be IFN-γ-independent[46]. Our DEG results showed that expression levels of IL-6, IL6ST, and their downstream targets, i.e., PIM3 and various cytokines, were significantly increased in P6-RNAi HEECs. Although we did not observe significant differences in expression levels in IFNγ and IFNγ receptor between P6-RNAi and control HEECs, the expression levels of eighteen IFN related genes were significantly increased, as well as the expression levels of downstream targets (OAS1, OAS2, OAS3, OASL, HLA-A) of IFNγ signaling in P6-RNAi HEECs. We speculate that IFNγ and IFNγ receptor expression levels might be transiently increased after Prdx6 knockdown but returned to normal levels by the time when we performed our measurements. Additional time course studies need to be performed to determine if IFNγ and IFNγ receptor expression levels are transiently increased after Prdx6 knockdown. Nevertheless, our results clearly demonstrated a relationship between Prdx6 and JAK1 / STAT1 signaling pathway in HEECs.

In addition, we tried to reveal how Prdx6 regulate JAK1 / STAT1 signaling pathway activation. Prdxs play an important role in reducing several cellular peroxide substrates, such as ROS[47]. Although high levels of ROS may result in oxidative stress to promote apoptosis in lymphocytes[48], certain amount of ROS is required to maintain cellular homeostasis[49]. Leong et al. (2000) in their paper described the immunomodulatory effects of ROS, in which ROS acts as a second messenger to participate in lymphocyte activation and as a third signal in T cell activation[50]. Our results showed that the MDA level and total antioxidant capacity in Prdx6-RNAi HEECs was significantly increased and decreased, respectively. Therefore, it can be speculated that knockdown of Prdx6 resulted in higher levels of ROS in
HEECs, which in turn, activated the IL-6 receptor and IFNγ to induce the JAK1 / STAT1 signaling pathway. However, the specific underlying molecular mechanisms need to be further investigated.

**Conclusion**

We speculated that knockdown of Prdx6 resulted in higher levels of ROS in HEECs, which in turn, activated the JAK1 / STAT1 signaling pathway induced by IL-6 receptor and IFN.

**Declarations**

**Funding**

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**Conflicts of interest/Competing interests**

The authors declare that they have no competing interests.

**Availability of data and material**

Not applicable.

**Code availability**

Not applicable.

**Authors’ contributions**

Hui Shi performed transfections, Western blotting experiments, DGE analysis, and drafted the manuscript. Xiaoyu Liu performed RT-PCR experiments and participated in DGE analysis. Yanwei Wang performed the cell culture experiments. Haiyan Wang performed statistical analysis. Bochen Pan and Jianyuan Li were involved in the design, coordination and helped to draft the manuscript. All authors have read and approved the final version of the manuscript, and agreed with the order of the authorships.

**Ethics approval**

Not applicable.
Consent to participate
Not applicable.

Consent for publication
Not applicable.

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Tables
### Table 1
The significantly different pathways

| Pathway                                      | Qvalue | Pathway ID |
|----------------------------------------------|--------|------------|
| 1 Hepatitis C                                | 0.001  | ko05160    |
| 2 Rheumatoid arthritis                       | 0.009  | ko05323    |
| 3 Toll-like receptor signaling pathway       | 0.021  | ko04620    |
| 4 Ribosome                                   | 0.021  | ko03010    |
| 5 Cytosolic DNA-sensing pathway              | 0.026  | ko04623    |
| 6 Cell cycle                                 | 0.026  | ko04110    |
| 7 NOD-like receptor signaling pathway        | 0.026  | ko04621    |
| 8 Cytokine-cytokine receptor interaction     | 0.026  | ko04060    |
| 9 Chagas disease (American trypanosomiasis) | 0.026  | ko05142    |
| 10 Osteoclast differentiation                | 0.026  | ko04380    |
| 11 RIG-I-like receptor signaling pathway     | 0.026  | ko04622    |
| 12 Ubiquitin mediated proteolysis            | 0.026  | ko04120    |
| 13 Other glycan degradation                  | 0.028  | ko00511    |
Table 2
The expressions of JAKs and STATs

| Gene symbol | log$_2$ Ratio (P6-RNAi / control cell) | P-Value | FDR | significant difference |
|-------------|----------------------------------------|---------|-----|------------------------|
| JAK1        | 1.34                                   | 8.28226E-14 | 2.6014E-12 | *                      |
| JAK2        | 1.35                                   | 0.01359128  | 0.045822224 | -                      |
| JAK3        | -1.94                                  | 0.001423868 | 0.006742904 | -                      |
| JAKMIP3     | -5.906890596                           | 0.2498   | 0.419522358 | -                      |
| STAT1       | 1.02                                   | 2.1736E-12 | 4.39471E-11 | *                      |
| STAT2       | 1.12                                   | 0.1156098  | 0.245160301 | -                      |
| STAT3       | -0.09                                  | 0.728464  | 0.827680221 | -                      |
| STAT4       | -0.42                                  | 0.725936  | 0.828291933 | -                      |
| STAT5A      | 1.19                                   | 0.0641106  | 0.154290407 | -                      |
| STAT5B      | -0.45                                  | 0.0708682  | 0.166866442 | -                      |
| STAT6       | 0.83                                   | 0.00974278 | 0.03466438  | -                      |
| SOCS1       | 0.485426827                            | 0.58175  | 0.720043576 | -                      |
| SOCS2       | -0.599324599                           | 0.00657522 | 0.0251786 | -                      |
| SOCS3       | -1.605721061                           | 7.16074E-07 | 6.9378E-06 | *                      |
| SOCS4       | 0.426804556                            | 0.0877092  | 0.197829067 | -                      |
| SOCS5       | -0.237136417                           | 0.419994  | 0.576760444 | -                      |
| SOCS6       | 0.358265781                            | 0.207484  | 0.370426821 | -                      |
| SOCS7       | 0.502753645                            | 0.0282318  | 0.08299928  | -                      |

P-Value: indicates the probability that a gene is expressed equally between two samples.

FDR: false discovery rate.

*: represents a significant difference between P6-RNAi and control cell groups.

-: represents no significant difference between P6-RNAi and control cell groups.
Table 3
The significantly different gene expressions related to IL-6

| Gene symbol | log\(_2\) Ratio (P6-RNAi / cell) | P-Value | FDR           | Description                                      |
|-------------|---------------------------------|---------|---------------|-------------------------------------------------|
| IL-6        | 3.08                            | 8.81672E-07 | 8.43333E-06  | interleukin-6 precursor                          |
| IL6ST       | 1.21                            | 1.86073E-13 | 5.11834E-12  | interleukin-6 receptor subunit beta isoform 3 precursor |
| PIM-3       | 1.61                            | 1.94098E-05 | 0.000148023 | threonine-protein kinase pim-3                   |
| CXCL10      | 8.71                            | 6.12788E-05 | 0.000417574 | C-X-C motif chemokine 10 precursor               |
| CXCL11      | 9.91                            | 2.34882E-10 | 3.66318E-09 | C-X-C motif chemokine 11 precursor               |
| CCL20       | 9.23                            | 9.5901E-07  | 9.10857E-06  | C-C motif chemokine 20 isoform 1                 |
| CCL2        | 3.17                            | 6.55254E-13 | 1.49744E-11 | C-C motif chemokine 2 precursor                 |
| CXCL3       | 2.93                            | 3.16648E-09 | 4.2176E-08  | C-X-C motif chemokine 3                          |
| CCL4        | 9.43                            | 1.19972E-07 | 1.30567E-06 | C-C motif chemokine 4 isoform 1 precursor        |
| CXCL1       | 2.99                            | 1.27232E-13 | 3.75194E-12 | growth-regulated alpha protein precursor         |
| CXCL2       | 2.92                            | 1.03249E-11 | 1.87682E-10 | C-X-C motif chemokine 2                          |
| CCL5        | 4.01                            | 1.67203E-09 | 2.33772E-08 | beta-chemokine RANTES precursor                 |
| IL8         | 2.48                            | 1.25233E-13 | 3.70921E-12 | interleukin 8                                   |
| IL1A        | 2.81                            | 5.68782E-05 | 0.000390146 | interleukin-1 alpha proprotein                  |
| IL17RA      | 1.32                            | 2.43992E-09 | 3.31192E-08 | interleukin-17 receptor A precursor              |
| IL15        | 1.24                            | 1.26275E-06 | 1.177E-05   | interleukin-15 isoform 1 preproprotein          |
| Gene symbol | log<sub>2</sub> Ratio (P6-RNAi / cell) | P-Value     | FDR          | Description                      |
|-------------|-------------------------------------|-------------|--------------|----------------------------------|
| IL4I1       | 2.43                                | 9.41942E-05 | 0.00061488   | L-amino-acid oxidase isoform 2   |
Table 4
The significantly different gene expressions related to IFNγ

| Gene symbol | \( \log_2 \) Ratio (P6-RNAi / cell) | P-Value       | FDR            | Description                                                                 |
|-------------|------------------------------------|---------------|----------------|----------------------------------------------------------------------------|
| IFRD1       | 1.04                               | 4.20214E-07   | 4.21025E-06    | interferon-related developmental regulator 1 isoform 1                     |
| IRF9        | 1.03                               | 3.04816E-11   | 5.25108E-10    | interferon regulatory factor 9                                              |
| IFI6        | 5.39                               | 0             | 0              | interferon alpha-inducible protein 6 isoform a                             |
| IFI27       | 5.07                               | 1.03249E-11   | 1.87935E-10    | interferon alpha-inducible protein 27, mitochondrial isoform 2             |
| MX1         | 4.46                               | 4.58758E-05   | 0.000319052    | interferon-induced GTP-binding protein Mx1                                 |
| IFI44       | 4.31                               | 4.6491E-10    | 6.97675E-09    | interferon-induced protein 44                                               |
| IFI44L      | 4.29                               | 8.81672E-07   | 8.41545E-06    | interferon-induced protein 44-like                                          |
| IFIT1       | 3.93                               | 2.77556E-13   | 7.12675E-12    | interferon-induced protein with tetratricopeptide repeats 1 isoform 2       |
| IFIT3       | 3.92                               | 4.996E-14     | 1.68269E-12    | interferon-induced protein with tetratricopeptide repeats 3                |
| IFITM1      | 3.29                               | 2.19824E-14   | 7.7721E-13     | interferon-induced transmembrane protein 1                                 |
| IFIT2       | 2.81                               | 0             | 0              | Interferon-induced protein with tetratricopeptide repeats 2                 |
| IFIH1       | 2.81                               | 7.4848E-08    | 8.41713E-07    | interferon-induced helicase C domain-containing protein 1                   |
| IFIT5       | 1.64                               | 2.5313E-14    | 8.87993E-13    | interferon-induced protein with tetratricopeptide repeats 5                |
| IFI30       | 1.56                               | 1.4997E-05    | 0.00011708     | gamma-interferon-inducible protein precursor                               |
| IFITM3      | 1.46                               | 8.88178E-14   | 2.75131E-12    | interferon-induced transmembrane protein 3                                 |
| GBP1        | 1.4                                | 6.01984E-07   | 5.90443E-06    | interferon-induced guanylate-binding protein 1                              |
| IFI16       | 1.22                               | 8.68612E-10   | 1.26281E-08    | Interferon, gamma-inducible protein 16 variant                             |
| IRF7        | 1.17                               | 2.98494E-12   | 5.89395E-11    | interferon regulatory factor 7 isoform d                                   |
| Gene symbol | log$_2$ Ratio (P6-RNAi / cell) | P-Value | FDR | Description |
|------------|-------------------------------|---------|-----|-------------|
| OAS1       | 3.85                          | 0.000027594 | 0.000203764 | 2'-5'-oligoadenylate synthase 1 isoform 3 |
| OAS2       | 11.17                         | 4.4409E-16 | 1.80117E-14 | 2'-5'-oligoadenylate synthase 2 isoform 3 |
| OAS3       | 3.71                          | 7.4848E-08 | 8.42414E-07 | 2'-5'-oligoadenylate synthase 3 |
| OASL       | 1.71                          | 0        | 0   | 59 kDa 2'-5'-oligoadenylate synthase-like protein isoform b |
| HLA-A      | 1.23                          | 2.51284E-11 | 4.39617E-10 | HLA class I histocompatibility antigen, A-1 alpha chain precursor |

Table 5
Target sequence of interference for human Prdx 6

| shRNA name     | Position on CDS | Target sequence                                      | GC content (%) |
|----------------|-----------------|------------------------------------------------------|----------------|
| si Prdx6       | 667             | agctggcaccagaatggccaaag                                 | 52             |
| irrelevant (control) | | agctagcactagaatctgcagag                                 | 52             |
| Gene name | Primer(5’-3’)                      | Tm (°C) |
|-----------|-----------------------------------|---------|
| GAPDH     | F: AACGGATTTGGCTGATTG; R: GGAAGATGGGTGATGGGATT | 51.5    |
| ICAM1     | F: TAGCAGCCGCAGCTCTATAA; R: AGAAAAGTGGGCAGGGAG | 53      |
| IL6       | F: GTCCAGTTGCTCTTCTCCC; R: GCCTCTTTTGCTGCTTTCA | 53.7    |
| BIRC3     | F: TGGTGGTATGTGCTCTGTA; R: TGGAAAAGTGCTCCTGGAGT | 48.1    |
| TSNAX     | F: TTAATATCGTGCCAAGCC; R: CCTCGTGATCTGCTACC | 49      |
| CCL5      | F: CCCTCGCTGCATCTCCTCA; R: CCCTCGCTGCATCCTCA  | 56.1    |
| IDO1      | F: CTGGAACTGCCTCCTATT; R: ATGCGAAGAACAATCAGAAA | 49.5    |
| TLR4      | F: GACCTGTCCCTGAACCCTA; R: AATATGTTGCCATCCGAAA | 49      |
| EDN2      | F: ACTTGGACATCATCTGGGTG; R: GAGGCTTTGACTGTGGAAA | 57      |
| MTOR      | F: AAAACCTCGTCATTTACCTAC; R: CAGCGAGTTCTTGGTCATTC | 54.1    |
| CCL2      | F: TGCTTCCCCCTCTACCTT; R: TGGATGTTCTGGTAGT | 51.1    |
| ASNS      | F: CTTCTGAGGGAAACTCTATT; R: AGCTGACTTTGTAGTGGAAT | 49.1    |
| MMP14     | F: CATCATTGAGGTGGACGAG; R: CATCATTGAGGTGGACGAG | 56.9    |
| FOSL      | F: ATTCAAAATCGCCCTGTG; R: ATGCGTGTTCTCTCCTCC | 53.1    |
| PPIA      | F: TTTGCAGAACAAGTCTCCA; R: TGGCCATCCAACCACTCA | 52.4    |
| BYSL      | F: GGGAGCAATCTCTACG; R: CACAGCACAGGCAGCTCA | 54      |
| GAMT      | F: CCTGCGCTGACGGTGACCT; R: CACAGACAGGCAGCTCA | 54      |
| UCP2      | F: GCTGGAGGTTGGTCGGAGAT; R: GGAGGCGATGACAGTGGT | 55.4    |
| WNT7B     | F: TAGACACCCTCTGTTTCCTT; R: TAGACACCCTCTGTTTCCTT | 56      |
| SHC1      | F: GGGAGAGGATAACCTGAAA; R: TGGCAACCATAGGGCGACAT | 53.8    |
| SPATA20   | F: AGTCACCACCTAACCCTACACCCA; R: TCCTCAGGTCTACCTCCAC | 53.7    |
| Prdx6     | F: AATTGGCCAAGAGGATG; R: GTGGTAGCTGGGGTAGAGG | 50.8    |

Tm: the melting temperature

Figures
Expression levels of PRDX6 protein and Prdx6 mRNA (A-C) and differentially expressed genes between control and P6-RNAi HEECs (D). Compared to control HEECs, the expression level of PRDX6 protein significantly decreased in P6-RNAi HEECs (A-B). The expression level of Prdx6 mRNA in P6-RNAi HEECs were reduced by 43.2% compared to control HEECs (C). Compared to control HEECs, there were 589 up-regulated genes and 314 down-regulated genes (including Prdx6) in P6-RNAi HEECs (D).
Evaluation of Sequencing Quality. The distribution patterns of total and distinct tags were similar between P6-RNAi and control HEECs. Tags with copy numbers > 100 were dominant in both control and P6-RNAi HEECs, while the majority of distinct tags had copy numbers < 5.
Figure 3

The differentially expressed genes measured by real-time PCR. Twenty genes were randomly selected to do real-time PCR for validation. Consistency was observed between real-time PCR and DGE results.

Figure 4

GO functional classification of the DEGs. Y-axis represents GO terms. All GO terms were grouped based on biological process, molecular function, and cellular component. The numbers in the bars represent DEGs annotated for biological process, molecular function, and cellular component. The percentage in the round brackets represent DEGs with relative annotation / All genes with relative annotation.
Figure 5

Western blot analysis of JAK1, STAT1, and SOCS3 expression levels (A) and gray degree analysis (B). Compared to control HEECs, the expression levels of JAK1, phosphorylated JAK1, STAT1, and phosphorylated STAT1 were significantly increased, while expression levels of SOCS3 were significantly reduced in P6-RNAi HEECs.

Figure 6

The MDA levels (A) and total antioxidant capacity (B) of HEECs. The MDA level in Prdx6-RNAi HEECs was significantly increased compared to that of control. The total antioxidant capacity, meanwhile, showed the opposite trend (Figure 6).