# Microarray analysis of differentially expressed genes in L929 mouse fibroblast cells exposed to leptin and hypoxia

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**Abstract.** Leptin and hypoxia are pro-fibrotic factors involved in fibrogenesis, however, the gene expression profiles remain to be fully elucidated. The aim of the present study was to investigate the regulatory roles of leptin and hypoxia on the L929 mouse fibroblast cell line. The cells were assigned to a normoxia, normoxia with leptin, hypoxia, and hypoxia with leptin group. The cDNA expression was detected using an Agilent mRNA array platform. The differentially expressed genes (DEGs) in response to leptin and hypoxia were identified using reverse transcription-quantitative polymerase chain reaction analysis, followed by clustering analysis, Gene Ontology analysis and pathway analysis. As a result, 54, 1,507 and 1,502 DEGs were found in response to leptin, hypoxia and the two combined, respectively, among which 52 (96.30%), 467 (30.99%) and 495 (32.96%) of the DEGs were down-regulated. The most significant functional terms in response to leptin were meiosis I for biological process (P=0.0041) and synaptonemal complex for cell component (P=0.0013). Only one significant pathway responded to leptin, which was axon guidance (P=0.029). Flow cytometry confirmed that leptin promoted L929 cell proliferation. The most significant functional terms in response to hypoxia were ion binding for molecular function (P=7.8621E-05), glucose metabolic process for biological process (P=0.0008) and cell projection part for cell component (P=0.003). There were 12 pathways, which significantly responded to hypoxia (P<0.05) and the pathway with the highest significance was the chemokine signaling pathway (P=0.0001), which comprised 28 genes, including C-C motif ligand (CCL)1, C-X-C motif ligand (CXCL)9, CXCL10, son of sevenless homolog 1, AKT serine/threonine kinase 2, Rho-associated protein kinase 1, vav guanine nucleotide exchange factor 1, CCL17, arrestin β1 and C-C motif chemokine receptor 2. In conclusion, the present study showed that leptin and hypoxia altered the profiles of gene expression in L929 cells. These findings not only extend the cell spectrum of leptin on cell proliferation, but also improve current understanding of hypoxia in fibroblast cells.

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## Introduction

Tissue fibrosis alters the tissue architecture and leads to organ dysfunction, which is major contributor to morbidity and mortality rates worldwide (1). The progression of fibrosis is similar in different organs, which is characterized by the activation and abnormal proliferation of fibroblasts/myofibroblasts and extracellular matrix remodeling (2). However, the mechanism underlying fibrogenesis is complex. Infection with pathogenic organisms, epigenetic alterations, B cells, transforming growth factor (TGF)-β signaling and TGFβ/small mothers against decapentaplegic (SMAD) 33-independent mechanisms have been reported to be involved in the activation of myofibroblasts (3-7). Several exogenous factors are also involved in fibrogenesis, including leptin and hypoxia (8-10). Previous studies have shown that leptin stimulates the production of tissue inhibitor of metalloproteinase 1 via the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway to directly promote fibrogenesis in hepatic stellate cells (11). Liver fibrosis is decreased in leptin- or leptin receptor-deficient mice (12). The *in vitro* administration of leptin to primary cardiofibroblasts has been found to result in the significant stimulation of pro-collagen Iα and also leads to a decrease in the gene expression of pro-matrix metalloproteinase-8, -9 and -13 at 24 h, which results in heart fibrosis (13). In addition, leptin is involved in renal fibrosis (14). Hypoxia is also an established profibrotic factor (9,15,16). In hepatic fibrosis, hypoxia acts as a major inducer of angiogenesis together with inflammation, and hepatic angiogenesis and fibrosis have been found to be closely associated in clinical and experimental conditions (8).

Hypoxia was found to induce cardiac fibrosis by upregulating focal adhesion kinase in cardiac fibroblasts or in a mouse model of post-myocardial infarction (17). Hypoxia-induced deoxycytidine kinase contributes to epithelial proliferation in pulmonary fibrosis (18). Hypoxia is also involved in hepatic fibrosis through potentiating the activity of hypoxia inducible factor-1α, either directly or through the epidermal growth factor (EGF)/mitogen-activated protein kinase (MAPK) and vascular endothelial growth factor (VEGF)/AKT pathway (8). However, the effects of leptin and hypoxia on fibrosis remain to be fully elucidated. The aim of the present study was to investigate the gene expression profiles of leptin and hypoxia in mouse fibroblast cell line L929 and analyze their possible biological functions in fibrosis processes. The present study showed that leptin and hypoxia altered the profiles of gene expression in L929 cells. The pro-fibrotic roles of leptin may be through promoting L929 cell proliferation; whereas hypoxia affected L929 cell function primarily through the chemokine signaling pathway.

## Materials and methods

*Cell culture and treatment*. The L929 mouse fibroblast cells, purchased from the Kunming Cell Bank (Kunming, China) were cultured in Dulbecco's modified Eagle's medium with 5% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a humidified 5% $CO_2$ incubator at 37˚C. The L929 cells were used for all the following experiments. For leptin treatment, mouse recombinant leptin (200 ng/ml; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) was added to the cells. For hypoxic treatment, the L929 cells were transferred to a hypoxia chamber (MIC101; Billups-Rothenberg, Inc., Del Mar, CA, USA) where the total oxygen concentration was reduced to <1%.

*cDNA expression array*. The cells were cultured in 10 cm plates with $2.5x10^6$ cells and divided into the following four groups: Group I, cells cultured in normoxia; Group II, cells treated with leptin in normoxia; Group III, cells cultured in hypoxia; Groups IV, cells treated with leptin in hypoxia. Every group included three parallel samples and the treatment temperature was 37˚C. After 24 h, the cells were collected and placed in TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), respectively.

Total RNA was extracted using TRIzol reagent according to the manufacturer's protocol. The RNA was purified using the mirVana miRNA isolation kit (Ambion; Thermo Fisher Scientific, Inc.). The RNA quality from each sample was assessed by visualization of the 28S/18S ribosomal RNA ratio using 1% formaldehyde denaturing gel electrophoresis. The Agilent mouse mRNA array was designed with eight identical arrays per slide (8x60K format), with each array containing probes interrogating ~39,430 Entrez Gene RNAs. The array also contained 1,280 Agilent control probes. The arrays were hybridized in an Agilent hybridization oven overnight at a rotation speed of 40 g at 42˚C and washed with two consecutive solutions (0.2% SDS, 2X SSC at 42˚C for 5 min and 0.2X SSC for 5 min at room temperature).

The array data were analyzed for data summarization, normalization and quality control using GeneSpring software V12 (Agilent; Thermo Fisher Scientific, Inc.) (19). To select the differentially expressed genes (DEGs), threshold values of ≥2 and ≤-2-fold change (FC) and a P-value of 0.05 were used. The data was $Log_2$-transformed and median centered by genes using the Adjust Data function of Cluster 3.0 software (www.falw.vu/~huik/cluster.htm), and then further analyzed by hierarchical clustering with average linkage (20). Finally, tree visualization was performed using Treeview (Stanford University School of Medicine, Stanford, CA, USA) (21).

*Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis*. The DEGs regulated by leptin and hypoxia identified by the microarray, were verified using RT-qPCR analysis. In total, five genes [Arrestin β1 (Arrb1), C-C motif ligand (Ccl)1, G protein-coupled receptor kinase 4 (Grk)4, Ccl17 and C-C motif chemokine receptor 2 (Ccr2)] were selected. Total RNA was extracted and the quality was assessed, as described above. The first-strand cDNA was synthesized using 500 ng total RNA in a 20.0 µl final volume by reverse transcription utilizing PrimeScript™ RT Master mix (Perfect Real Time; Takara Bio, Inc., Otsu, Japan). Subsequently, the cDNA was diluted in five volumes sterile water. The qPCR was performed in a volume of 20.0 µl using 2.0 µl cDNA, 0.8 µl specific forward primer, 0.8 µl specific reverse primer, 10.0 µl SYBR® Select Master mix (Thermo Fisher Scientific, USA) and 6.4 µl deionized water. The amplification was performed using a Roche LightCycler® detection system (Roche Diagnostics, Indianapolis, IN, USA). The primers (Sangon Biotech Co, Ltd., Shanghai, China) were as follows: Arrb1, forward 5'-AGG CAT CAC TGG ATA AGG AG-3' and reverse 5'-GTC TTG TTG GTG TTG TTG GTG-3'; Ccl1, forward 5'-TTC CCC TGA AGT TTA TCC AG-3' and reverse 5'-GAT TTT GAA CCC ACG TTT TG-3'; Grk4, forward 5'-ATG GAG GGG ATT TGA AGT AC-3' and reverse 5'-CTG GCT TTA GGT CTC TGT AT-3'; Ccl17, forward 5'-GCT GCC TGG ATT ACT TCA AAG-3' and reverse 5'-TTT GTC TTT GGG GTC TGC AC-3'; Ccr2, forward 5'-TGT AGT CAC TTG GGT GGT GG-3' and reverse 5'-TAA GGG CCA CAG GTG TAA TG-3'. For all RT-qPCR experiments, negative controls comprised a non-reverse transcription reaction and a non-sample reaction (data not shown). Actin was amplified as an internal standard. The $2^{-\Delta\Delta Cq}$ method was applied for data analysis (22).

*Functional enrichment analysis*. The Database for Annotation, Visualization, and Integrated Discovery (DAVID) is widely used in functional enrichment analysis of DEGs (23). In the present study, DAVID (david.abcc.ncifcrf.gov) was used to perform functional enrichment analysis for the DEGs regulated by leptin, hypoxia and the two combined, respectively. The genes were mapped to Gene Ontology (GO) terms for this purpose. The GO annotation (www.geneontology.org) provides a descriptive framework and functional annotation of DEGs, and is comprised of biological processes, cellular components and molecular functions. In addition, Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/) pathway enrichment analysis was performed to map the potential pathways of the DEGs (24). The P-value cut-off associated with this analysis was set at $P<0.05$ in order to identify significantly enriched functional terms and pathways.

*Cell cycle analysis using flow cytometry (FCM).* The cells were seeded at a density of $10x10^4$ per well in six-well plates in triplicate and allowed to adhere for 24 h. Following starvation, the cells were treated with or without leptin (200 ng/ml) in normoxic conditions at 37˚C. Following culture for 24 h, the cells were harvested and fixed in 70% cold ethanol at -20˚C overnight. The cells were stained with propidium iodide (Sigma-Aldrich; Merck Millipore) at 50 $\mu$g/ml with 20 $\mu$g/ml RNase A at room temperature in the dark for 1 h prior to analysis. The cell population fraction in each phase of the cell cycle was determined as a function of the DNA content using FCM (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA). Data analysis was performed using FlowJo v10 software (Tree Star, Inc., Ashland, OR, USA) (25). This experiment was repeated three times.

*Statistical analysis.* Values are presented as the mean ± standard deviation unless otherwise indicated using SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA). Statistical analysis was performed using Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Microarray analysis and hierarchical clustering.* The genes induced in the cultured L929 cells by leptin, hypoxia and the two combined were analyzed using a cDNA array. The array included four groups containing 12 samples. The cluster results of the four sets of microarray data are shown in Fig. 1. The two primary gene clusters were identified visually based on the heat map signal intensity in groups I and II, vs. groups III and IV. The expression of genes in cluster 1 were higher in groups I and II, compared with that in groups III and IV, suggesting that those genes may be suppressed by hypoxia. By contrast, cluster 2 consisted of genes activated by hypoxia. It appeared that leptin was a weak factor affecting gene profiling in normoxia and hypoxia.

The genes with FC values >2.0 and $P<0.05$ were considered to be a DEG. In the present study, 54 DEGs were found in the leptin-treated group, of which 52 (96.30%) were downregulated. A total of 1,507 DEGs were found under hypoxia treatment, of which 467 (30.99%) were downregulated. In the group treated with leptin and hypoxia, 1,502 DEGs were found, among which 495 (32.96%) were downregulated. However, compared with the hypoxia group, there were only 11 genes altered in the leptin and hypoxia treatment group, comprising three (27.27%) downregulated and eight (72.73%) upregulated genes.

*Verification of array data using RT-qPCR analysis.* To assess the reliability of the array data, five genes (Arrb1, Ccl1, Grk4, Ccl17 and Ccr2) were selected for amplification, with normoxia and hypoxia samples as a template, using RT-qPCR analysis. The $2^{-\Delta\Delta Cq}$ method was used for the determination of target mRNA following normalizing of target mRNA Cq values with those for actin (ΔCq). In the array data, two genes (Arrb1 and Ccl1) were upregulated in the hypoxia samples by 3.51- and 6.78-fold, respectively. In the RT-qPCR experiments, the upregulated FC values were 2.78- and 5.44-fold, respectively (Fig. 2). The other three genes (Grk4, Ccl17 and Ccr2) were downregulated in the hypoxia samples, by 2.11-, 2.11- and 2.15-fold, respectively, and the FC values in the RT-qPCR experiments were 1.92-, 3.19- and 2.87-fold, respectively (Fig. 2). These results suggested that the array data was in correspondence with the RT-qPCR experiments.

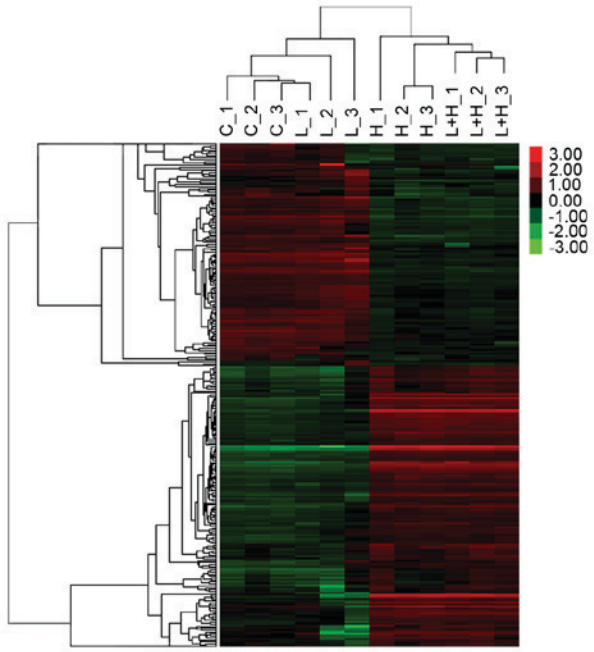


Figure 1. Array data. Hierarchical clustering dendrogram comparing leptin-altered, hypoxia-altered, and leptin and hypoxia-treated groups to normoxia control exposure. Each sample listed contains the average gene expression value for three replicates. C, normoxia control; L, leptin 200 ng/ml; H, hypoxia, 1% O2; L+H, leptin and hypoxia (200 ng/ml leptin and 1% O2). Green, low expression; red, high expression.

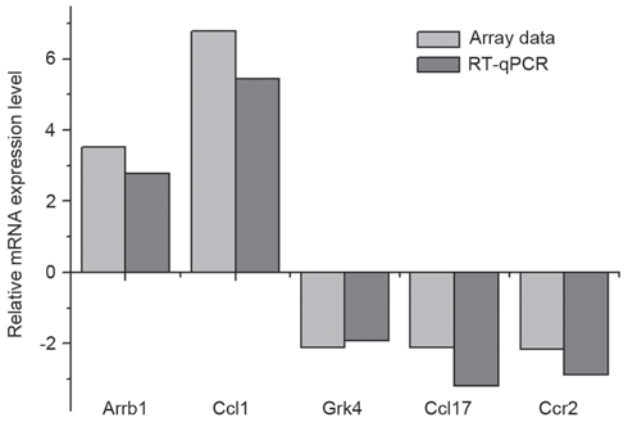


Figure 2. RT-qPCR verification of the array data. Fold changes of selected genes in normoxia and hypoxia groups using microarray and RT-qPCR analyses. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; Arrb1, Arrestin β1; Ccl1, C-C motif ligand 1, Grk4, G protein-coupled receptor kinase 4; Ccl17, C-C motif ligand 17; Ccr2, C-C motif chemokine receptor 2.

*Functional enrichment analysis.* To investigate the biological roles of the DEGs regulated by leptin, hypoxia and the two combined in L929 cells, a categorized GO enrichment analysis was performed, comprising 54, 1,507 and 1,502 genes, respectively (Tables I-III). For the DEGs response to leptin, meiosis

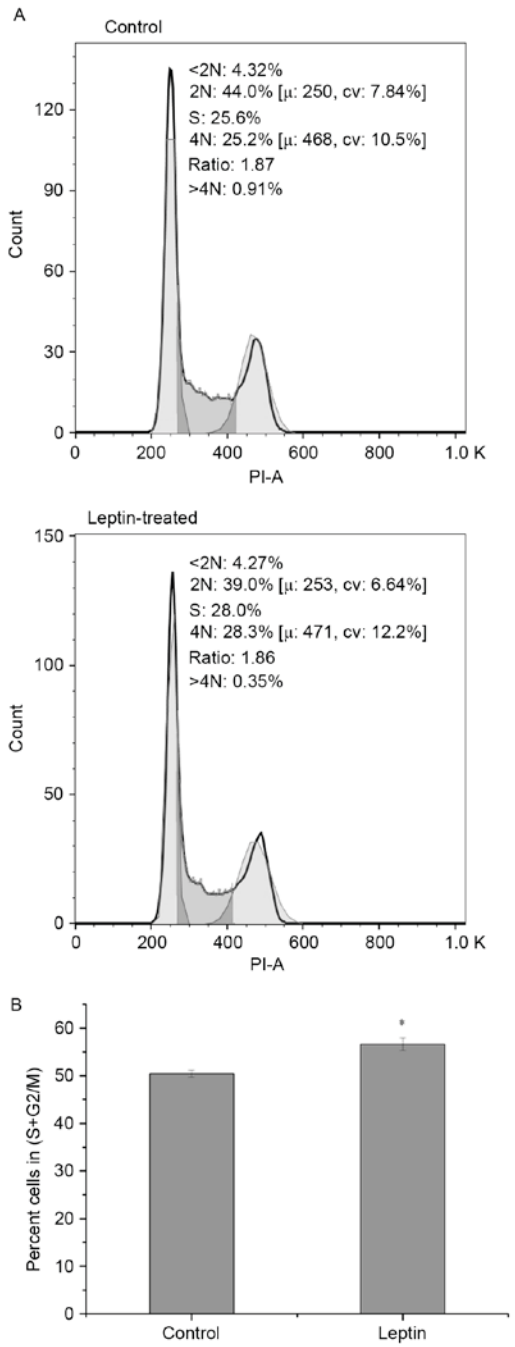


Figure 3. Effects of leptin on cell cycle progression in L929 cells. The cells were treated with 200 ng/ml leptin for 24 h, and (A) DNA content was analyzed using fluorescence flow cytometry following PI staining. (B) Percentages of cells in the S+G2/M phase. PI, propidium iodide. *P=0.033.

I (P=0.004) and synaptonemal complex (P=0.001) were the most significantly enriched functional terms for biological processes and cellular components, respectively. For the DEGs response to hypoxia, glucose metabolic process (P=0.0008), cell projection part (P=0.003) and ion binding (P=7.8621E-05) were the most significantly enriched functional terms for biological processes, cellular components and molecular functions, respectively. For the DEGs regulated by leptin and hypoxia combined, phosphate metabolic process (P=0.0007) and extracellular region (P=0.0022) were the most significantly enriched functional terms for biological processes and cellular components, respectively.

Table I. GO analysis for the differentially expressed genes regulated by leptin.

| Term | Genes (n) | P-value |
|---|---|---|
| Molecular function | - | - |
| Biological process | | |
| GO:0007127 meiosis I | 3 | 0.004056677 |
| GO:0022402 cell cycle process | 5 | 0.021448529 |
| GO:0051327 M phase of meiotic cell cycle | 3 | 0.023847468 |
| GO:0007126 meiosis | 3 | 0.023847468 |
| GO:0007049 cell cycle | 6 | 0.023981901 |
| GO:0051321 meiotic cell cycle | 3 | 0.024865727 |
| GO:0007129 synapsis | 2 | 0.045322159 |
| GO:0070192 chromosome organization involved in meiosis | 2 | 0.045322159 |
| Cell component | | |
| GO:0000795 synaptonemal complex | 3 | 0.001322361 |
| GO:0044454 nuclear chromosome part | 4 | 0.002698438 |
| GO:0000793 condensed chromosome | 4 | 0.003006214 |
| GO:0000228 nuclear chromosome | 4 | 0.004250276 |
| GO:0000794 condensed nuclear chromosome | 3 | 0.005772372 |
| GO:0044427 chromosomal part | 5 | 0.010435104 |
| GO:0005694 chromosome | 5 | 0.018637296 |
| GO:0000800 lateral element | 2 | 0.018883838 |

GO, Gene Ontology.

*Pathway enrichment analysis*. KEGG pathway enrichment analysis was performed to assess the biological roles of the DEGs (Table IV). Axon guidance was the only significant pathway in response to leptin (P=0.0294). There were 12 significant pathways in response to hypoxia, among which the chemokine signaling pathway (P=0.00014) was the most significant, which suggested inflammatory factors were crucial in the response to hypoxia in L929 cells. For the combined treatment group, nine pathways were significant, of which eight were identical to the responses to hypoxia: Nitrogen metabolism, focal adhesion, chemokine signaling pathway, arginine and proline metabolism, starch and sucrose metabolism, pyruvate metabolism, VEGF signaling pathway and MAPK signaling pathway.

*Leptin promotes the proliferation of L929 cells*. The results of the functional enrichment analysis suggested that leptin affected the cell cycle progression of L929 cells under hypoxia. To confirm this, the numbers of cells in different cell cycle phases were detected using FCM. Exposing the L929 cells to leptin resulted in a high percentage of cells in the S+G2/M

Table II. GO analysis for the differentially expressed genes regulated by hypoxia.

| Term | Genes (n) | P-value |
|---|---|---|
| Molecular function | | |
| GO:0043167 ion binding | 333 | 7.8621E-05 |
| GO:0043169 cation binding | 329 | 8.79225E-05 |
| GO:0046872 metal ion binding | 326 | 9.99094E-05 |
| GO:0016836 hydro-lyase activity | 12 | 0.000122629 |
| GO:0017016 Ras GTPase binding | 12 | 0.001984074 |
| GO:0000287 magnesium ion binding | 46 | 0.002246305 |
| GO:0031267 small GTPase binding | 12 | 0.002642921 |
| GO:0016702 oxidoreductase activity, acting on single donors with incorporation of molecular oxygen, incorporation of two atoms of oxygen | 13 | 0.002791662 |
| GO:0016701 oxidoreductase activity, acting on single donors with incorporation of molecular oxygen | 13 | 0.003165874 |
| GO:0051020 GTPase binding | 12 | 0.003951419 |
| GO:0046914 transition metal ion binding | 218 | 0.005063268 |
| GO:0019899 enzyme binding | 28 | 0.006555908 |
| GO:0005506 iron ion binding | 38 | 0.007222115 |
| GO:0001882 nucleoside binding | 135 | 0.009641824 |
| GO:0001883 purine nucleoside binding | 134 | 0.010743224 |
| GO:0031418 L-ascorbic acid binding | 6 | 0.011357513 |
| GO:0004725 protein tyrosine phosphatase activity | 15 | 0.011975484 |
| GO:0030554 adenyl nucleotide binding | 132 | 0.01339165 |
| GO:0003779 actin binding | 32 | 0.013513591 |
| GO:0004674 protein serine/threonine kinase activity | 43 | 0.016010492 |
| GO:0031406 carboxylic acid binding | 13 | 0.016733784 |
| GO:0004672 protein kinase activity | 56 | 0.018393253 |
| GO:0008092 cytoskeletal protein binding | 42 | 0.019021647 |
| GO:0005524 ATP binding | 123 | 0.022617503 |
| GO:0019992 diacylglycerol binding | 10 | 0.022656776 |
| GO:0019842 vitamin binding | 16 | 0.024555951 |
| GO:0015293 symporter activity | 17 | 0.02501501 |
| GO:0017076 purine nucleotide binding | 154 | 0.029861653 |
| GO:0032559 adenyl ribonucleotide binding | 123 | 0.030297935 |
| GO:0016641 oxidoreductase activity, acting on the CH-NH2 group of donors, oxygen as acceptor | 5 | 0.034869252 |
| GO:0004089 carbonate dehydratase activity | 5 | 0.034869252 |
| GO:0050662 coenzyme binding | 19 | 0.035408286 |
| GO:0004721 phosphoprotein phosphatase activity | 18 | 0.042027399 |
| GO:0016723 oxidoreductase activity, oxidizing metal ions, NAD or NADP as acceptor | 3 | 0.043728093 |
| GO:0008138 protein tyrosine/serine/threonine phosphatase activity | 7 | 0.049875355 |
| Biological process | | |
| GO:0006006 glucose metabolic process | 22 | 0.000792317 |
| GO:0055114 oxidation reduction | 70 | 0.000950229 |
| GO:0006796 phosphate metabolic process | 83 | 0.003423909 |
| GO:0006793 phosphorus metabolic process | 83 | 0.003423909 |
| GO:0044271 nitrogen compound biosynthetic process | 35 | 0.004517996 |
| GO:0007601 visual perception | 16 | 0.004646543 |
| GO:0050953 sensory perception of light stimulus | 16 | 0.00510266 |
| GO:0007242 intracellular signaling cascade | 85 | 0.006819851 |
| GO:0019318 hexose metabolic process | 22 | 0.007922216 |
| GO:0005996 monosaccharide metabolic process | 24 | 0.008183748 |
| GO:0044275 cellular carbohydrate catabolic process | 11 | 0.008669622 |

Table II. Continued.

| Term | Genes (n) | P-value |
|---|---|---|
| GO:0006468 protein amino acid phosphorylation | 62 | 0.009474011 |
| GO:0006096 glycolysis | 9 | 0.010826957 |
| GO:0008015 blood circulation | 16 | 0.011053616 |
| GO:0003013 circulatory system process | 16 | 0.011053616 |
| GO:0001666 response to hypoxia | 11 | 0.013524164 |
| GO:0070482 response to oxygen levels | 11 | 0.015005602 |
| GO:0042384 cilium assembly | 6 | 0.016566907 |
| GO:0007010 cytoskeleton organization | 34 | 0.022567205 |
| GO:0051241 negative regulation of multicellular organismal process | 14 | 0.023152487 |
| GO:0030029 actin filament-based process | 21 | 0.0232384 |
| GO:0030036 actin cytoskeleton organization | 20 | 0.023432887 |
| GO:0048545 response to steroid hormone stimulus | 10 | 0.023828973 |
| GO:0019374 galactolipid metabolic process | 3 | 0.02707365 |
| GO:0006681 galactosylceramide metabolic process | 3 | 0.02707365 |
| GO:0019320 hexose catabolic process | 9 | 0.028066811 |
| GO:0006007 glucose catabolic process | 9 | 0.028066811 |
| GO:0006470 protein amino acid dephosphorylation | 15 | 0.029397775 |
| GO:0006767 water-soluble vitamin metabolic process | 7 | 0.029717956 |
| GO:0051046 regulation of secretion | 16 | 0.029739379 |
| GO:0016310 phosphorylation | 65 | 0.030184101 |
| GO:0016265 death | 49 | 0.03112936 |
| GO:0060271 cilium morphogenesis | 6 | 0.032808209 |
| GO:0009743 response to carbohydrate stimulus | 6 | 0.032808209 |
| GO:0006778 porphyrin metabolic process | 6 | 0.032808209 |
| GO:0033013 tetrapyrrole metabolic process | 6 | 0.032808209 |
| GO:0009967 positive regulation of signal transduction | 20 | 0.034149817 |
| GO:0046365 monosaccharide catabolic process | 9 | 0.034329668 |
| GO:0060341 regulation of cellular localization | 19 | 0.034742883 |
| GO:0009719 response to endogenous stimulus | 21 | 0.035172676 |
| GO:0006730 one-carbon metabolic process | 15 | 0.035666401 |
| GO:0042403 thyroid hormone metabolic process | 4 | 0.037600598 |
| GO:0046164 alcohol catabolic process | 10 | 0.03779069 |
| GO:0016311 dephosphorylation | 17 | 0.039806519 |
| GO:0006357 regulation of transcription from RNA polymerase II promoter | 56 | 0.04088076 |
| GO:0008219 cell death | 47 | 0.046075704 |
| GO:0006955 immune response | 44 | 0.047117585 |
| GO:0048660 regulation of smooth muscle cell proliferation | 5 | 0.04805859 |
| GO:0050873 brown fat cell differentiation | 6 | 0.049834676 |
| Cell component | | |
| GO:0044463 cell projection part | 23 | 0.003220028 |
| GO:0042995 cell projection | 58 | 0.003517983 |
| GO:0005886 plasma membrane | 234 | 0.004494904 |
| GO:0005576 extracellular region | 143 | 0.00502389 |
| GO:0031225 anchored to membrane | 25 | 0.008680624 |
| GO:0019898 extrinsic to membrane | 46 | 0.017185716 |
| GO:0044441 cilium part | 8 | 0.020951644 |
| GO:0042579 microbody | 14 | 0.026699591 |
| GO:0005777 peroxisome | 14 | 0.026699591 |
| GO:0044421 extracellular region part | 68 | 0.031279026 |
| GO:0045121 membrane raft | 12 | 0.031670056 |
| GO:0043005 neuron projection | 26 | 0.032261958 |
| GO:0019897 extrinsic to plasma membrane | 9 | 0.034092369 |

Table II. Continued.

| Term | Genes (n) | P-value |
|---|---|---|
| GO:0005829 cytosol | 50 | 0.038894078 |
| GO:0005930 axoneme | 7 | 0.039557367 |
| GO:0005901 caveola | 6 | 0.04056632 |
| GO:0031672 A band | 4 | 0.045209466 |
| GO:0005929 cilium | 16 | 0.045846025 |
| GO:0042598 vesicular fraction | 20 | 0.046732261 |
| GO:0005741 mitochondrial outer membrane | 11 | 0.048199094 |
| GO:0030016 myofibril | 12 | 0.048557001 |

GO, Gene Ontology.

Table III. GO analysis for the differentially expressed genes regulated by leptin and hypoxia.

| Term | Genes (n) | P-value |
|---|---|---|
| Molecular function | - | - |
| Biological process | | |
| GO:0006796 phosphate metabolic process | 87 | 0.00069201 |
| GO:0006793 phosphorus metabolic process | 87 | 0.00069201 |
| GO:0016265 death | 57 | 0.000914867 |
| GO:0006468 protein amino acid phosphorylation | 67 | 0.001122958 |
| GO:0017157 regulation of exocytosis | 9 | 0.001368273 |
| GO:0008219 cell death | 55 | 0.001517519 |
| GO:0055114 oxidation reduction | 68 | 0.002418208 |
| GO:0012501 programmed cell death | 51 | 0.002614104 |
| GO:0044271 nitrogen compound biosynthetic process | 35 | 0.004597316 |
| GO:0006915 apoptosis | 49 | 0.004983028 |
| GO:0016310 phosphorylation | 70 | 0.005034959 |
| GO:0003016 respiratory system process | 4 | 0.005961782 |
| GO:0009743 response to carbohydrate stimulus | 7 | 0.008246776 |
| GO:0006006 glucose metabolic process | 19 | 0.009614291 |
| GO:0051241 negative regulation of multicellular organismal process | 15 | 0.010348645 |
| GO:0007601 visual perception | 15 | 0.011259001 |
| GO:0032940 secretion by cell | 23 | 0.011798862 |
| GO:0050953 sensory perception of light stimulus | 15 | 0.012230633 |
| GO:0046903 secretion | 26 | 0.013035463 |
| GO:0001666 response to hypoxia | 11 | 0.013616099 |
| GO:0070482 response to oxygen levels | 11 | 0.015106629 |
| GO:0009746 response to hexose stimulus | 6 | 0.016635896 |
| GO:0001974 blood vessel remodeling | 6 | 0.016635896 |
| GO:0009749 response to glucose stimulus | 6 | 0.016635896 |
| GO:0034284 response to monosaccharide stimulus | 6 | 0.016635896 |
| GO:0006730 one-carbon metabolic process | 16 | 0.01753354 |
| GO:0009719 response to endogenous stimulus | 22 | 0.019740633 |
| GO:0048608 reproductive structure development | 17 | 0.020659779 |
| GO:0048545 response to steroid hormone stimulus | 10 | 0.023972008 |
| GO:0003013 circulatory system process | 15 | 0.024166653 |
| GO:0008015 blood circulation | 15 | 0.024166653 |
| GO:0001775 cell activation | 27 | 0.025299039 |
| GO:0007242 intracellular signaling cascade | 81 | 0.02535934 |
| GO:0006865 amino acid transport | 11 | 0.026707116 |

Table III. Continued.

| Term | Genes (n) | P-value |
|---|---|---|
| GO:0006681 galactosylceramide metabolic process | 3 | 0.027127455 |
| GO:0019374 galactolipid metabolic process | 3 | 0.027127455 |
| GO:0005996 monosaccharide metabolic process | 22 | 0.028503233 |
| GO:0019318 hexose metabolic process | 20 | 0.029456322 |
| GO:0006470 protein amino acid dephosphorylation | 15 | 0.029624933 |
| GO:0045944 positive regulation of transcription from RNA polymerase II promoter | 36 | 0.030944318 |
| GO:0006778 porphyrin metabolic process | 6 | 0.032936937 |
| GO:0033013 tetrapyrrole metabolic process | 6 | 0.032936937 |
| GO:0003006 reproductive developmental process | 28 | 0.033137813 |
| GO:0003001 generation of a signal involved in cell-cell signaling | 12 | 0.033562678 |
| GO:0009967 positive regulation of signal transduction | 20 | 0.034460687 |
| GO:0046324 regulation of glucose import | 5 | 0.040773001 |
| GO:0006357 regulation of transcription from RNA polymerase II promoter | 56 | 0.041404785 |
| GO:0045893 positive regulation of transcription, DNA-dependent | 40 | 0.041795439 |
| GO:0006979 response to oxidative stress | 12 | 0.041889132 |
| GO:0051254 positive regulation of RNA metabolic process | 40 | 0.045730238 |
| GO:0009220 pyrimidine ribonucleotide biosynthetic process | 4 | 0.047696549 |
| GO:0009218 pyrimidine ribonucleotide metabolic process | 4 | 0.047696549 |
| GO:0010827 regulation of glucose transport | 5 | 0.048213767 |
| GO:0042398 cellular amino acid derivative biosynthetic process | 8 | 0.049928789 |
| Cell component | | |
| GO:0005576 extracellular region | 145 | 0.002250808 |
| GO:0044463 cell projection part | 23 | 0.003001467 |
| GO:0005777 peroxisome | 16 | 0.00482117 |
| GO:0042579 microbody | 16 | 0.00482117 |
| GO:0042995 cell projection | 55 | 0.011330557 |
| GO:0045121 membrane raft | 13 | 0.013007776 |
| GO:0031225 anchored to membrane | 24 | 0.015194373 |
| GO:0005886 plasma membrane | 227 | 0.016114278 |
| GO:0008021 synaptic vesicle | 11 | 0.017112432 |
| GO:0044456 synapse part | 24 | 0.019656437 |
| GO:0033267 axon part | 6 | 0.025413 |
| GO:0044421 extracellular region part | 68 | 0.028509841 |
| GO:0019898 extrinsic to membrane | 44 | 0.035509102 |
| GO:0005730 nucleolus | 31 | 0.035643165 |
| GO:0043232 intracellular non-membrane-bounded organelle | 152 | 0.036036538 |
| GO:0043228 non-membrane-bounded organelle | 152 | 0.036036538 |

GO, Gene Ontology.

phases, which indicated that leptin promoted L929 cell proliferation (Fig. 3A and B).

## Discussion

Investigations have increasingly focused on the pro-fibrotic microenvironment of organs. Leptin and hypoxia are pro-fibrotic factors, which are involved in fibrogenesis. In the present study, a high-throughput microarray method was applied to detect the expression profile response to leptin, hypoxia and the two combined in L929 cells. It was found that leptin promoted mouse fibroblast cell proliferation, whereas hypoxia affected L929 cell function, primarily through the chemokine signaling pathway.

The present study identified 54 leptin-responsive genes, 52 of which were downregulated >2-fold. Among these, nephronophthisis 3, also known as pcy, showed a marked reduction by 3.3-fold. It has been reported that pcy mice undergoing

Table IV. Pathway analysis of the differentially expressed genes regulated by leptin, hypoxia and the two in combination.

| KEGG ID | Term | n | P-value |
|---|---|---|---|
| Leptin-treated | | | |
| mmu04360 | Axon guidance | 3 | 0.029392665 |
| Hypoxia-treated | | | |
| mmu04062 | Chemokine signaling pathway | 28 | 0.00014053 |
| mmu00500 | Starch and sucrose metabolism | 10 | 0.000638251 |
| mmu00910 | Nitrogen metabolism | 7 | 0.004127542 |
| mmu00052 | Galactose metabolism | 7 | 0.009539235 |
| mmu04510 | Focal adhesion | 24 | 0.010772098 |
| mmu00380 | Tryptophan metabolism | 8 | 0.019533919 |
| mmu04360 | Axon guidance | 17 | 0.020000767 |
| mmu00620 | Pyruvate metabolism | 8 | 0.022185656 |
| mmu00330 | Arginine and proline metabolism | 9 | 0.0297123 |
| mmu04010 | MAPK signaling pathway | 28 | 0.029963278 |
| mmu04370 | VEGF signaling pathway | 11 | 0.03808443 |
| mmu00010 | Glycolysis/gluconeogenesis | 10 | 0.046234215 |
| Leptin and hypoxia-treated | | | |
| mmu00910 | Nitrogen metabolism | 7 | 0.004076684 |
| mmu04510 | Focal adhesion | 25 | 0.005345589 |
| mmu04062 | Chemokine signaling pathway | 23 | 0.007744979 |
| mmu00330 | Arginine and proline metabolism | 10 | 0.010268331 |
| mmu00500 | Starch and sucrose metabolism | 8 | 0.010980612 |
| mmu00620 | Pyruvate metabolism | 8 | 0.021915205 |
| mmu04630 | JAK-STAT signaling pathway | 18 | 0.035405855 |
| mmu04370 | VEGF signaling pathway | 11 | 0.037534097 |
| mmu04010 | MAPK signaling pathway | 27 | 0.047450835 |

KEGG, Kyoto Encyclopedia of Genes and Genomes; MAPK, mitogen-activated protein kinase; VEGF, vascular endothelial growth factor; JAK, Janus kinase; STAT, signal transducer and activator of transcription.

cystogenesis present with progressive increasingly severe renal fibrosis (26). Another gene, E2f transcription factor 4 (E2f4), showed a marked reduction by 3.0-fold. E2f4 is important in the suppression of proliferation-associated genes and is involved in the G1/S phase of the mitotic cell cycle (27-30). This may account for the percentage of cells in the G1/S phase being decreased and that in the S+G2/M being increased in response to leptin. Leptin-stimulated cell proliferation had been reported previously, including in vascular smooth muscle cell proliferation (31), hepatic stellate cells (32) and cancer cells (33).

Pathway analysis revealed the significant pathway regulated by leptin was axon guidance, of which three genes, Eph receptor A5, Rho-associated coiled-coil containing protein kinase 1 (Rock1) and semaphoring 6D, were significantly affected. These results were concordant with previous studies. A study by Simerly (34) found that leptin may direct the development of hypothalamic pathways by promoting axonal projections. A study by Harrold (35) indicated novel regulatory roles for leptin in synaptic plasticity and axon guidance.

Several genes varied in response to hypoxia. The most significant pathway response to hypoxia was the chemokine signaling pathway, and the expression of 28 genes (Ccl1, adenylate cyclase 4, protein kinase C, G protein subunit α1, Cxcl9, G protein subunit γ (Gng)13, Cxcl10, dedicator of cytokinesis 2, son of sevenless homolog 1, Gng2, phosphoinositide-3-kinase regulatory subunit 3, phospholipase Cβ2, SHC adaptor protein 2, AKT serine/threonine kinase 2, Gng7, mitogen-activated protein kinase kinase 1, Rock1, vav guanine nucleotide exchange factor 1, Ccl17, engulfment and cell motility 1, Arrb1, glycogen synthase kinase 3β, Ccr2, G protein subunit β5, RAP1A, member of RAS oncogene family, Grk4, Jak3 and Crk) were altered in this pathway. Among these, Ccr2 and CC chemokine ligand 2 (Ccl2) receptor were previously reported to be altered in oxygen shortage (36). In addition, Cxcl9, Cxcl10 and Ccl17 have been reported to be involved in the pathogenesis of lung fibrosis (37). This result further suggested that inflammation was important in L929 function, particularly in pathological states. Therefore, it was hypothesized that the hypoxic microenvironment facilitates L929 cell proliferation through the chemokine signaling pathway, and the uncontrolled inflammation further promotes fibrosis. Further understanding of the mechanisms involved in chemokine-mediated cell proliferation may lead to improved therapeutic strategies in fibrosis.

Several other pathways were involved in the response to hypoxia, including starch and sucrose metabolism, nitrogen

metabolism and galactose metabolism. These pathways associated to metabolism were in accordance with expectations, as cell adaptation to low oxygen concentrations involves repression of mitochondrial respiration and induction of glycolysis to sustain cell function in hypoxic conditions (38). Axon guidance was also a significant pathway response to hypoxia and to leptin, which was coincident with a previous study (39). Therefore, under hypoxia, several pathways may function in concert to restore oxygen supply to cells and modulate cell function to adapt the hypoxic conditions.

In conclusion, the present study showed that leptin and hypoxia altered gene expression profiles in L929 cells. The results suggested that the pro-fibrotic effects of leptin may be through promoting mouse fibroblast cell proliferation; whereas hypoxia affected mouse fibroblast cell function predominantly through the chemokine signaling pathway. These findings improve understanding of leptin and hypoxia in fibroblast cells. Axon guidance and the chemokine signaling pathway may represent novel therapeutic targets for leptin and hypoxia injury in fibrogenesis, and require further investigation.

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