

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 β 1 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.

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 TGF β /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 I α 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).

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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-1 α , 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% CO₂ 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 2.5x10⁶ 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 Log₂-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 β 1 (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 μ l 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 μ l using 2.0 μ l cDNA, 0.8 μ l specific forward primer, 0.8 μ l specific reverse primer, 10.0 μ l SYBR® Select Master mix (Thermo Fisher Scientific, USA) and 6.4 μ l 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^{- $\Delta\Delta$ C_q} 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 10×10^4 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 µg/ml with 20 µg/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

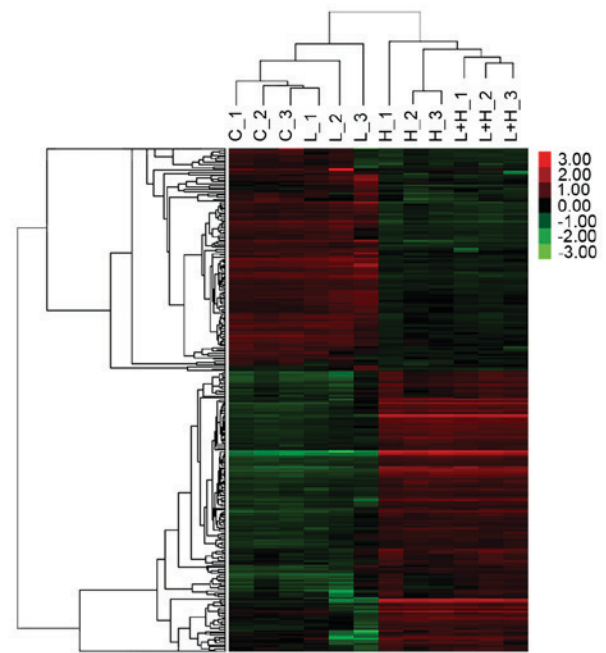


Figure 1. Array data. Hierarchical clustering dendrogram comparing leptin-altered, hypoxia-altered, and leptin and hypoxia-treated groups to normoxia control exposure. Each sample listed contains the average gene expression value for three replicates. C, normoxia control; L, leptin 200 ng/ml; H, hypoxia,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

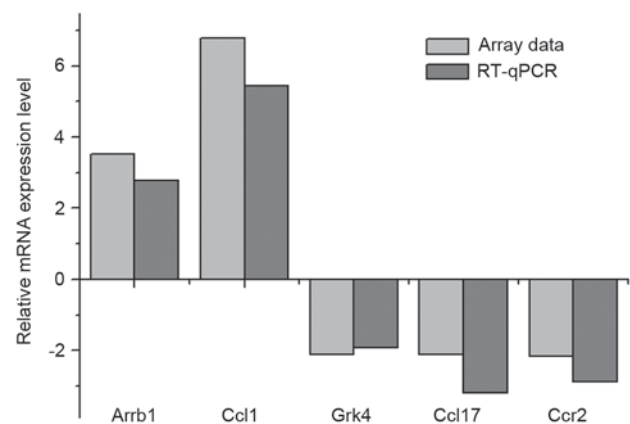


Figure 2. RT-qPCR verification of the array data. Fold changes of selected genes in normoxia and hypoxia groups using microarray and RT-qPCR analyses. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; Arrb1, Arrestin β 1; Ccl1, C-C motif ligand 1; Grk4, G protein-coupled receptor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

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.

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

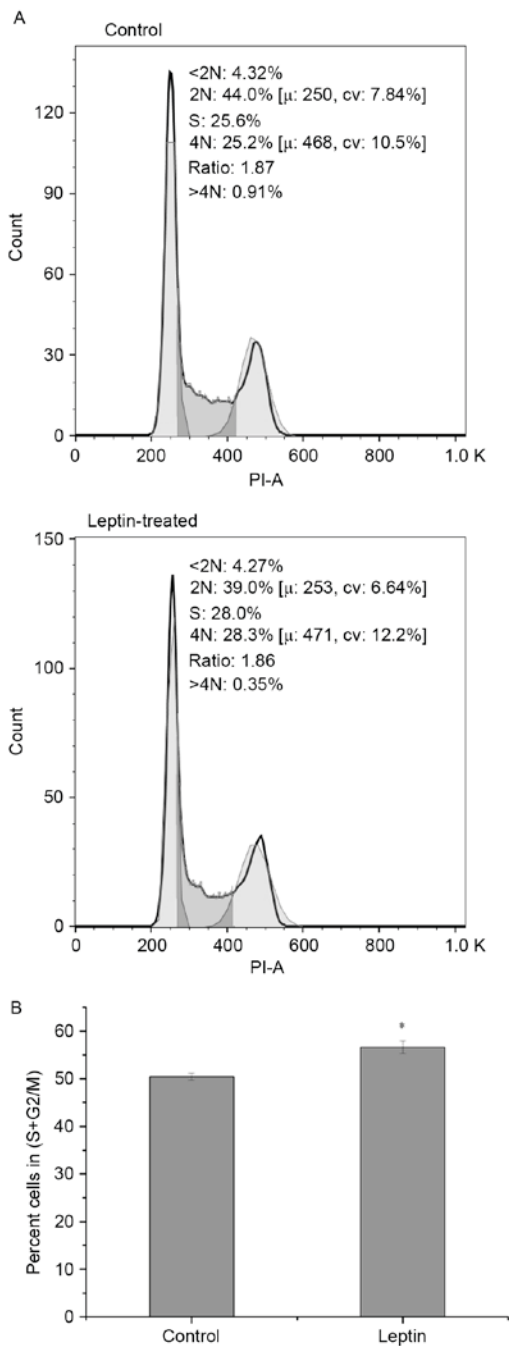


Figure 3. Effects of leptin on cell cycle progression in L929 cells. The cells were treated with 200 ng/ml leptin for 24 h, and (A) DNA content was analyzed using fluorescence flow cytometry following PI staining. (B) Percentages of cells in the S+G2/M phase. PI, propidium iodide. * $P=0.033$.

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 adenylyl 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 adenylyl 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, nephropththisis 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

$\alpha 1$, 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 C $\beta 2$, 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 3 β , Ccr2, G protein subunit $\beta 5$, 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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