Leptin Induces Cyclin D1 Expression and Proliferation of Human Nucleus Pulposus Cells via JAK/STAT, PI3K/Akt and MEK/ERK Pathways

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Abstract

Increasing evidence suggests that obesity and aberrant proliferation of nucleus pulposus (NP) cells are associated with intervertebral disc degeneration. Leptin, a hormone with increased circulating level in obesity, has been shown to stimulate cell proliferation in a tissue-dependent manner. Here, we show that leptin induced the proliferation of primary cultured human NP cells, which expressed the leptin receptors OBRa and OBRb. Induction of NP cell proliferation was confirmed by CCK8 assay and immunocytochemistry and Real-time PCR for PCNA and Ki-67. Mechanistically, leptin induced the phosphorylation of STAT3, Akt and ERK1/2 accompanied by the upregulation of cyclin D1. Pharmacological inhibition of JAK/STAT3, PI3K/Akt or MEK/ERK signaling by AG490, Wortmannin or U0126, respectively, reduced leptin-induced cyclin D1 expression and NP cell proliferation. These experiments also revealed an intricate cross talk among these signaling pathways in mediating the action of leptin. Taken together, we show that leptin induces human NP cell cyclin D1 expression and proliferation via activation of JAK/STAT3, PI3K/Akt or MEK/ERK signaling. Our findings may provide a novel molecular mechanism that explains the association between obesity and intervertebral disc degeneration.

Introduction

The high morbidity of low back pain causes severe incapacity that increases medical expense and impacts the workforce, posing high socioeconomic costs [1]. Effective treatment of low back pain is therefore a matter of great public concern. Although the etiology of low back pain is multifactorial, intervertebral disc degeneration (IVD) is thought to be a major cause [2]. IVD is a process that is influenced by genetic predisposition, lifestyles (e.g. occupation, smoking, alcohol consumption), co-morbidities (e.g. obesity and diabetes), and aging [3]. Several biomechanical parameters, such as height, fluid pressurization, dissipation, stiffness, and flexibility, are implicated in the initiation and progression of IVD [4]. Other factors, such as formation of cell cluster and the proliferation of fibrocartilaginous tissue, may also take part in IVD [5]. Thus far, the cause of increased cell proliferation in IVD remains unclear.

First described in 1994, leptin (the 16 kDa product of the OB gene) is a peptide hormone secreted mainly by adipose tissues [6]. It is also produced by a variety of cells including placental cells and gastric epithelial cells [7]. Fibrocartilaginous tissues, including articular cartilage and intervertebral disc, have been recently recognized as other sources of leptin [8]. Serum leptin levels are positively associated with body weight, implicating the involvement of this hormone in the regulation of food intake [9]. In addition, leptin is implicated in the modulation of other physiological processes, such as angiogenesis, wound healing, central and peripheral endocrine actions, and renal and pulmonary functions [10]. Emerging evidence suggest that leptin may function as a growth factor to stimulate cell proliferation in a tissue-dependent manner [11]. For instance, exogenous leptin induces sustained proliferative responses in prostate and lung epithelial cells, pancreatic beta cells as well as breast and gastric cancer cells [12]. A recent study has shown that human herniated disc tissues and rat NP cells express leptin and its functional receptor [13]. Leptin also stimulates the proliferation of rat NP cells in vitro [14]. Nevertheless, it remains unclear whether leptin can induce human NP cell proliferation. Moreover, the mechanism of leptin-induced NP cell proliferation has not yet been elucidated in human or animals.

Leptin exerts its action through its cell membrane receptor that belongs to class I cytokine receptor family. Thus far, six receptor isoforms, designated as OBRa to OBRf, have been identified [15]. However, only OBRb (the long isoform of the OBR) contains intracellular motifs required for the initiation of intracellular signaling [16]. OBRb mediates the action of leptin via multiple signaling pathways, including Janus kinase/signal transducers and activators of transcription (JAK/STAT), mitogen-activated protein kinases (MAPK), protein kinase C (PKC), nitric oxide (NO), and cyclic AMP pathways [17]. In other cell types, leptin also activates extracellular signal-regulated kinase 1/2 (ERK1/2) and phosphatidylinositol 3-kinase (PI3K) in a SHP-2- and IRS-dependent manner, respectively [18]. The complexity of leptin...
signaling is increased by the crosstalk among these signaling pathways. The involvement of these pathways in leptin signaling in NP cells remains unknown.

Obesity is an established risk factor for IVD in which leptin may play a significant role in its pathogenesis. In the present study, we determined the effect of leptin on the proliferation of primary cultured human NP cells and delineated the underlying molecular mechanism.

Results

Morphology, Immunofluorescence Characterization and Real-time Quantitative PCR Validation of Primary Cultured Human NP cells

After approximately 1 week, the primary cultured human NP cells reached almost complete confluence. The cells were polygonal, with multiple cytoplasmic processes and granular cytoplasm (Fig. 1A). Immunofluorescence for collagenase type II and cytokeratin 19 was observed in these cells. Figure 1C and 1D shows a typical field observed in these cultures. CA12, which was recently described by Minogue et al as a marker gene for human NP cells, was found to have significantly higher levels of expression in NP cells and NP tissue than in AF tissue and chondrocytes (Fig. 1D). IBSP and FBN1, which were also recently described by Minogue et al as negative markers for human NP cells, was found to have significantly low levels of expression in NP cells and NP tissue than in AF tissue and chondrocytes (Fig. 1E and 1F).

Human NP cells Expressed Leptin Receptors

The expression of two isoforms of leptin receptors, namely OBRa and OBRb, was determined in primary cultured human NP cells by RT-PCR and Real-time RT-PCR. Results indicated that the mRNA expression of both isoforms was detected in human NP cells (Fig. 2A and 2B), hinting at the possibility that NP cells are capable of receiving leptin signals, at least in part, via these two OBRs.

Leptin Induced Human NP cell Proliferation

In serum-replete conditions, increasing concentrations of leptin (1–1500 ng/ml) significantly increased NP cell proliferation in a dose-dependent manner, with the maximal response at 1000 ng/ml at 48 h (Fig. 2C). Time-dependent induction of NP cell proliferation by leptin (10 ng/ml), a concentration within the range of plasma concentrations found in obese individuals, was observed with the maximal response at 96 h (Fig. 2D). The prosurvival effect of leptin on the loss of NP cell viability induced by serum deprivation was also examined. In this set of experiments, NP cells were pre-incubated in serum-free medium for 1 day before leptin stimulation in which serum deprivation was continuously maintained. As shown in Figure 2E, serum deprivation reduced the number of viable NP cells in all groups. Nevertheless, the number of viable cells in the leptin-treated group was significantly higher than that of the serum-free group but was not significantly different from the serum-replete group. This finding suggests that, in addition to its proliferative effect, leptin may maintain NP cell survival in face of nutrient starvation. The proliferative effect of leptin was confirmed immunohistochemical staining of PCNA and Ki-67 in NP cells treated with or without leptin (Fig. 3A, 3B). As shown in Figure 3C, 3F, there was a significant increase in the percentage of PCNA-positive and Ki-67-positive NP cells in the group treated with 10 ng/ml leptin as compared with the control group. Treatment with leptin (10 ng/ml) significantly increased PCNA and Ki-67 mRNA level in a time-dependent manner, with a maximal response at 48 h or 24 h respectively (Fig. 3D, 3G).

Leptin Induced STAT3, Akt and ERK Phosphorylation in Human NP cells

Leptin has been shown to instigate intracellular signaling via activation of JAK2/STAT3, PI3K/Akt, and MEK/ERK pathways in other cell types. In the present study, the effect of leptin on the activities of these pathways in human NP cells was determined by Western blot for total and phosphorylated STAT3, Akt, and ERK1/2. As shown in Figure 4, leptin stimulation time-dependently increased the phosphorylation of STAT3, Akt and ERK1/2 without altering the total protein levels. The induction of STAT3, Akt and ERK1/2 phosphorylation could be observed as early as 5 min after leptin stimulation and the maximal stimulation occurred at 30 min, 15 min and 5 min post-stimulation, respectively. These findings indicate that leptin could readily activate JAK2/STAT3, PI3K/Akt, and MEK/ERK pathways in human NP cells.

Pharmacological Inhibition of JAK2/STAT3, PI3K/Akt, and MEK/ERK Pathways Prevented Leptin-induced NP cells Proliferation

To examine the possible involvements of JAK2/STAT3, PI3K/Akt, and MEK/ERK pathways in leptin-induced NP cell proliferation, NP cells were treated with or without AG490 (JAK inhibitor), wortmannin (PI3K inhibitor) or U0126 (MEK inhibitor), alone or in combination, in the absence or presence of leptin. Results from CCK8 cell proliferation assays showed that AG490 or U0126 remarkably reduced leptin-induced NP cell proliferation whereas wortmannin exerted only modest inhibition. However, co-inhibition of PI3K and MEK with wortmannin and U0126 could almost completely block the proliferative effect of leptin in NP cells as in other combined treatment groups (AG490+wortmannin, AG490+U0126, AG490+wortmannin+U0126). In contrast, these inhibitors per se did not significantly alter NP cell proliferation, indicating that inhibition of JAK2/STAT3, PI3K/Akt, and MEK/ERK pathways specifically blocked the proliferative effect of leptin (Fig. 5).

Crosstalk Among JAK/STAT3, PI3K/Akt, and MEK/ERK Pathways in Leptin-stimulated NP cells

The data presented so far indicates that JAK/STAT3, PI3K/Akt, and MEK/ERK pathways mediated the mitogenic effect of leptin in NP cells. Whether there is crosstalk among these three signaling pathways remained unclear. Western blot analysis indicates that U0126, AG490 and wortmannin significantly reduced leptin-induced ERK1/2, STAT3 and Akt phosphorylation, respectively. Interestingly, in addition to its effect on STAT3 phosphorylation, JAK2 inhibitor AG490 also partially reduced phosphorylation of ERK1/2 but not Akt induced by leptin. In contrast, MEK inhibitor U0126 reduced phosphorylation of ERK1/2 whereas wortmannin specifically reduced Akt phosphorylation induced by leptin (Fig. 6).

Leptin Induced Cyclin D1 Expression in a JAK-, PI3K-, and MEK-dependent Manner

Increased cyclin D1 expression is known to promote cell cycle progression during G1-S transition. Here we examined the possible involvement of cyclin D1 in leptin-induced NP cell proliferation and its relationship with the JAK/STAT3, PI3K/Akt, and MEK/ERK pathways. Western blot and Real-time RT-PCR analysis show that leptin time-dependently increase cyclin D1 protein and
Figure 1. Morphology, immunofluorescence characterization and real-time quantitative PCR validation of primary cultured human NP cells. (A) Phase-contrast photomicrograph of primary NP cells cultured in vitro for about 1 week, just before reaching complete confluence. Original magnification, ×40. (B) Real-time RT-PCR analysis of novel NP cell marker gene CA12 in NP cells, chondrocytes, NP and AF. Real-time RT-PCR analysis was performed in triplicate and the expression levels of CA12 mRNAs were normalized to GAPDH mRNAs. Error bars represent standard deviation. (C) Fluorescence microscopy images showing collagenase type II were observed in NP cells. Nuclei were stained with DAPI, shown in blue. Images were acquired using laser scanning confocal microscopy under a 40× objective. (D) Fluorescence microscopy images showing cytokeratin 19 were observed in NP cells. Nuclei were stained with DAPI, shown in blue. Images were acquired using laser scanning confocal microscopy under a 40× objective. (E) Fluorescence microscopy images showing IBSP (E) and FBN1 (F) in NP cells, chondrocytes, NP and AF. Real-time RT-PCR analysis was performed in triplicate and the expression levels of IBSP and FBN1 mRNAs were normalized to GAPDH mRNAs.
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mRNA expression in human NP cells, with the both maximal response at 72 h. Furthermore, inhibitors of JAK (AG490), PI3K (wortmannin) or MEK (U0126) blocked leptin-induced cyclin D1 protein and mRNA expression (Fig. 7).

**Discussion**

Increasing epidemiological evidence has supported that obesity is closely associated with IVD [19]. The cellular and molecular mechanism of obesity-related IVD, however, remains unclear. In this respect, leptin, a hormone with increased circulating levels in obese patients, has been implicated in the pathogenesis obesity-related IVD. We first characterized NP cells by assessing the morphology, the expression of collagenase type II, cytokeratin 19, CA 21, IBSP and FBN1. The results showed that NP cells in this study possessed the above these characteristics of NP cells, which was consistent with previous research [20,21]. In this study, we demonstrated that leptin directly stimulated proliferation of human NP cells which expressed leptin receptors OBRa and OBRb. The mitogenic effect of leptin was supported by CCK-8 assay, immunostaining for two proliferative markers PCNA and Ki-67 as well as induction of cyclin D1. In this regard, disc cell proliferation is known to be associated with IVD and is likely the cause of cluster formation. In fact, proliferating cells have been found to be defective in the synthesis of normal matrix components and thereby promoting disc degeneration. These findings suggest that increased NP cell proliferation induced by leptin may be one of the possible mechanisms underlying the detrimental influence of obesity on the development of IVD; on the other hand, the effect induced by leptin on NP cell may be fine for normal intervertebral disc.
Although leptin functions as a growth factor in various types of cells, the exact mechanism of leptin-induced cell proliferation is not fully understood. In the present study, we showed that leptin-induced NP cell proliferation was accompanied with increased phosphorylation of STAT3, Akt and ERK1/2 and upregulation of cyclin D1. Inhibition of JAK, PI3K or MEK also reduced leptin-induced NP cell proliferation.
induced cyclin D1 expression and NP cell proliferation. These findings suggest that leptin induces cyclin D1 expression and proliferation of human NP cells via JAK/STAT, PI3K/Akt and MEK/ERK pathways. Cyclin D1 is an important mediator that controls cell cycle transition from G1-to-S-phase [22]. In this regard, leptin has been shown to induce cyclin D1 in human breast and endometrial cancer cells as well as hepatocarcinoma cells [23–25]. To this end, the regulation of cyclin D1 and cell proliferation by JAK/STAT, PI3K/Akt and MEK/ERK signaling has been widely reported, especially in cancer biology studies [26]. Here, we demonstrate that the concomitant activation of JAK/STAT, PI3K/Akt and MEK/ERK pathways is required for induction of cyclin D1 and cell proliferation by leptin in human NP cells. In fact, other cytokines, such as PDGF, bFGF and IGF-I have been found to stimulate the proliferation of disc cells via the ERK and Akt signaling pathways and are implicated in the development of IVD [27].

Leptin acts via transmembrane receptors, which are structurally similar to the class I cytokine receptor family. Leptin receptor OBR is produced in several alternatively spliced forms designated OBRa to OBRf [15]. These OBRs are expressed in a variety of tissue including lung, kidney, liver, stomach and articular cartilage [28,29]. Among these OBRs, OBRb is the best studied and believed to be the major signal mediator of leptin. Activation of leptin receptors has been previously shown to stimulate JAK2/STAT3 pathway, PI3K/Akt and MEK/ERK pathways in other

Figure 4. Leptin activates phosphorylations of STAT3, Akt, and ERK1/2 in NP cells. After 1-day serum deprivation, leptin (10 ng/ml) was added into the serum-free medium of NP cells for 5 min, 15 min and 30 min, and then the protein amounts of phosphorylated forms of Akt (p-Akt) (A), ERK1/2 (p-ERK1/2) (B) or STAT3 (p-STAT3) (C) were detected with western blotting analysis. GAPDH was also detected for a loading control. doi:10.1371/journal.pone.0053176.g004
from this work is that there is a reciprocal regulation between JAK2/STAT3 and MEK/ERK pathways, in which U0126 (MEK inhibitor) abolished STAT3 phosphorylation while AG490 (JAK inhibitor) partially reduced ERK phosphorylation induced by leptin in human NP cells. This finding is in discrepancy with those reported by Trinko et al. showing that U0126 blocked leptin-induced phosphorylation of ERK1/2 but not STAT3 in the central nervous system of rats [31]. Yin et al. also showed that JAK inhibition but not MEK inhibition prevented the growth stimulation of breast cancer cells by leptin [32]. The mechanism underlying these discrepancies, however, warrants further investigation. One of the drawbacks of this study is the lack of age-matched non-degenerate discs as control. NP cells derived from LDD patients may not necessarily reflect the in vivo scenario. This study only evaluated the effect of leptin on LDD NP cells. The comparison of the reaction of LDD and normal NP cells to leptin stimulation should provide further information for the potential involvement of leptin in LDD development. In conclusion, the evidence presented in this work indicates that leptin stimulates proliferation of human NP cells. The mitogenic effect of leptin is mediated through upregulation of cyclin D1 via concomitant activation of JAK/STAT3, PI3K/Akt, or MEK/ERK pathways that may become potential targets for pharmacological intervention. Our data also reveal an unreported signaling crosstalk among JAK/STAT3, PI3K/Akt, or MEK/ERK pathways in mediating the action of leptin. These molecular alterations constitute a possible link between obesity and an increased risk for IVD.

### Materials and Methods

#### Ethics Statement

All of the experimental protocols were approved by the Clinical Research Ethics Committee of the Peking Union Medical College Hospital. Human lumbar IVD samples obtained from patients undergoing discectomy following approval from the Clinical Research Ethics Committee of the Peking Union Medical College Hospital and fully informed written consent of patients.

#### Reagents

Inhibitors were purchased from the following sources: JAK inhibitor AG490 (E)-2-Cyano-3-(3,4-dihydrophenyl)-N-[phenylmethyl]-2-propenamide), MEK inhibitor U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene) and PI3K inhibitor Wortmannin were purchased from Sigma. All other chemicals and reagents were purchased from Sigma unless otherwise specified.

#### Isolation and Primary Culture of Human NP cells

The human NP cells were dissected from patient disc surgical specimens (8 donors, 31–40 years, and average Thompson degeneration grade 2–3). All of these NP tissues was isolated from patients underwent surgeries for disc degeneration (L4/5) and not disc herniation, and therefore contact between these tissues and cells outside of the disc, these are macrophages, endothelial cells and other immune cells, were minimal or nonexistent. No granulation tissue was present. Two cell types populate the human NP: notochordal cells and chondrocyte-like cells [33]. As the notochordal cells slowly decline in abundance and appear to be absent after 10 years of age [34], NP tissues of human adolescents and adults consist of only chondrocyte-like cells. NP cells were isolated and cultured as previously described [35,36]. Tissues specimens were first washed thrice with PBS, NP was separated from the AF using a stereotaxic microscope, then cut into pieces (2–3 mm3), and NP cells were released from the NP tissues by
incubation with 0.25 mg/ml type II collagenase for 12 h at 37 °C in Dulbecco’s modified Eagle medium (DMEM; GIBCO, Grand Island, NY). After isolation, NP cells were resuspended in DMEM containing 10% FBS (GIBCO, NY, USA), 100 μg/ml streptomycin, 100 U/ml penicillin and 1% L-glutamine, and then incubated at 37 °C in a humidified atmosphere with 95% air and 5% CO2. The confluent cells were detached by trypsinization, seeded into 35-mm tissue culture dishes in complete culture medium (DMEM supplemented with 10% FBS, 100 μg/ml streptomycin and 100 U/ml penicillin) in a 37 °C, 5% CO2 environment. The medium was charged every 2 days. NP cells cultured in vitro within 10 days, the second passage was used for subsequent experiments.

Reverse Transcription (RT)-polymerase Chain Reaction (PCR) for Detection of OBRa/b

Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The RNA was quantified by absorbance at 260 nm. cDNA was synthesized from 2 μg of total RNA using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Oligo (dT)18 was used as the RT primer for the reverse transcription of mRNAs. Briefly, 2 μg total RNA with 1 μl dNTP and 1 μM Oligo (dT)18 in a total volume of 8 μl were denatured at 65°C for 5 min, immediately cooled on ice, and incubated with the reverse transcriptase reaction mixture supplemented with 1U RNase inhibitor in a total volume of 20 μl at 37°C for 60 min to generate the first-stand cDNA. The reaction was terminated by incubation at 55°C for 5 min and rapidly cooled on ice. The reverse-transcribed cDNA was further PCR-amplified by specific primers in 20 μl PCR mixture (cDNA, 0.25 mM NTP, 1 μM forward and reverse primers, 0.5 U Tap) for 35 cycles. The primer sets for detection of OBR and GAPDH are listed in Table 1.

Real-time PCR

Total mRNA was extracted from cells by using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer’s instructions. RNA was isolated with chloroform and isopropanol, washed with ethanol, and dissolved in water. Quantification of RNA was based on spectrophotometric analysis at 260/280 nm with values between 1.8 and 2.0 confirmed the purity of the RNA samples. A 2-μg sample of total RNA was reverse-transcribed with 200 U of MMLV reverse transcriptase (Invitrogen) using Oligo(dT) primers in a 20 μL reaction mixture following the manufacturer’s instructions. Relative transcript levels of OBRa, OBRb, CA21, Cyclin D1, PCNA and Ki-67 mRNA were determined by real-time PCR using the iQ5 Real-Time PCR Detection System (Bio-Rad, California, USA). The real-time PCR reaction was composed of 1x SYBR Green fluorescent dye (Takara, Dalian, China), 1 μM forward primers (10 μM), 1 μM reverse primers (10 μM), 1x qPCR mix,
The sequences of the specific primers are shown in Table 1. To produce the melting curve, the reactions were subject to one step at 95°C for 30 s followed by 45 cycles of 95°C for 5 s, 60°C for 10 s, and 72°C for 30 s. The relative gene expression was assessed by the \( \Delta \Delta Ct \) method. GAPDH was used as an internal control.

Figure 7. Leptin induces NP cells’ cyclin D1 expression and pharmacological inhibitors of JAK, PI3K/Akt, and MEK/ERK1/2 prevent NP cells’ cyclin D1 expression from leptin induction. (A) After 1-day serum deprivation, NP cells were incubated in serum-free media containing 10 ng/ml leptin for varying time intervals (12–96 h), and then the amounts of cyclin D1 protein were detected with western blotting analysis using GAPDH as an internal control. (B) NP cells were serum starved for 24 h, and then treated with vehicle (−), 10 ng/ml leptin (+), 10 \( \mu M \) U0126 (U0), 40 \( \mu M \) AG490 (AG) or 250 nM wortmannin (Wort) for 48 h. The amounts of cyclin D1 protein were detected with western blotting analysis using GAPDH as an internal control. (C) Real-time RT-PCR analysis of cyclin D1 mRNA expression in NP cells following leptin treatment for 0, 12, 24, 48 h, 72 h, or 96 h. Real-time RT-PCR analysis was performed in triplicate and the expression levels of cyclin mRNAs were normalized to GAPDH mRNAs. Error bars represent standard deviation. (D) NP cells were serum starved for 24 h, and then treated with vehicle (−), 10 ng/ml leptin (+), 10 \( \mu M \) U0126 (U0), 40 \( \mu M \) AG490 (AG) or 250 nM wortmannin (Wort) for 48 h. Cyclin D1 mRNA expression were detected with Real-time RT-PCR analysis using GAPDH as an internal control. Error bars represent standard deviation.

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Table 1. Nucleotide sequences of primers used in Real-time RT-PCR.

| Gene | Forward Primer | Reverse Primer | Tm (°C) | Product size (bp) |
|------|----------------|----------------|---------|------------------|
| CA12 | CTTGGCTGCTCTGTGGATCT | AGCCACCTGGAACCGTTCCT | 60      | 70               |
| OBRa | TTGGCCAGAATTTTATTCTCTT | AGTGGCAATATTGGTTCAT | 56      | 200              |
| OBRb | CCAGGAACCTTTCTGACATC | CAAAAGACACACCATCTCCTC | 56      | 609              |
| Cyclin D1 | AAC TACTCGGCACGCCTCTCT | CCACCTG GACCTGTCACCA | 56      | 204              |
| PCNA | AGTGAGAGAATGGAAGATGGAA | GAGACATGGAGCTTGTGTT | 56      | 154              |
| Ki-67 | TCTTTGTTGGGACCTGACACCTG | TGTGGTTGAGGTCCATCCATG | 56      | 156              |
| IBSP | CCAGGAGGAACATCACCAAA | GCACAGGCCATCCAAA | 60      | 68               |
| FBLN1 | CCTTGGAGCCGGGAGACTA | ACCGATGGCCTGACCA | 60      | 74               |
| GAPDH | TCAAGGACCACCTTTGCAAGCTCAGCT | GGTGGTCAGGGGTCTTAC | 56      | 116              |

Determination of NP cell Proliferation by Cell Counting Kit-8 (CCK8) Assay

NP cells were seeded in 96-well plates at the density of 1000 cells per well with 100 µl of complete culture medium. After adherence for 24 hours, the medium was changed to DMEM/F-12 supplemented with or without 5% (v/v) FBS and recombinant human leptin (Sigma-Aldrich, Oakville, ON, Canada) was added to the medium to final concentrations ranging from 1 ng/ml to 1000 ng/ml. The cells were then cultured for another 24, 48 or 96 h. Cells that did not exposed to leptin were used as controls and the wells to which only culture medium was added served as blanks. At the end of leptin stimulation, the supernatant was removed, and 100 µl of DMEM/F12 medium containing 10 µl of CCK8 was added to each well for another 3 h at 37°C. The culture plates were then shaken for 10 min and the optical density (OD) values were read at 450 nm.

Immunohistochemistry for Proliferative Markers PCNA and Ki-67

Coverslips were placed into 24-well plate and then NP cells were plated and treated with or without 10 ng/ml leptin for 48 h. Afterwards, medium was removed and the cells were washed twice with PBS and fixed with 3.5% formaldehyde for 30 min at 37°C. The cells were rinsed with PBS for 3 times, permeabilized with 0.1% (v/v) Triton X-100 in PBS for 20 min and blocked with 3% (w/v) BSA and 0.05% (v/v) Tween 20 in PBS for 30 min at room temperature. After blocking, the cells were incubated overnight at 4°C with primary antibody. The antibodies used were as follows: rabbit monoclonal anti-PCNA antibody (dilution ratio 1:100, Bioworldle, USA) and rabbit monoclonal anti-Ki-67 antibody (dilution ratio 1:500, Bioworldle, USA). The cells were then treated with fluorescent anti-rabbit secondary antibody (1:500, Bioworldle, USA) for 2 h at room temperature. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Fluorescence images were acquired with a Leica TCS SP2 confocal microscopy (Leica, Mannheim, Germany) using the Leica Confocal Software.

Western Blot Analysis for Cyclin D1 and Total and Phosphorylated STAT3, ERK1/2 and Akt

Western blot was performed as described with modifications [37]. Briefly, total cellular proteins were extracted from NP cells with the lysis buffer and separated on 10% SDS-PAGE gel. After electrophoresis, proteins were electrotransferred onto the nitrocellulose membrane (Millipore, MA, USA). The membrane was then blocked with 5% (w/v) skim milk in TBST (20 nM Tris-HCl(pH 7.6), 137 nM NaCl, and 0.1% Tween-20), probed with appropriate primary antibodies at 4°C overnight and bound with HRP-conjugated secondary antibodies at room temperature for 1 h. Chemiluminescent signal was detected using ECL kit (Millipore, MA, USA) and autoradiography.

Statistical Analysis

Results were expressed as means ± SD of multiple experiments. Statistical analysis was performed with Student’s t-test for comparison between two groups or an analysis of variance (ANOVA) followed by the Turkey’s t-test for comparison of multiple groups. P values less than 0.05 were considered statistically significant.

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Author Contributions

Conceived and designed the experiments: ZL, JS. Performed the experiments: ZL, XY. Analyzed the data: ZL, J. Liang, J. Liu. Contributed reagents/materials/analysis tools: ZL, JS, GQ. Wrote the paper: ZL, WKKW.

References

1. Katz JN (2006) Lumbar disc disorders and low-back pain: socioeconomic factors and consequences. J Bone Joint Surg Am 88 Suppl 2: 21–24.
2. Millecamps M, Tajerian M, Naoo L, Sage EH, Stone LS (2012) Lumbar intervertebral disc degeneration associated with axial and radiating low back pain in ageing SPARC-null mice. Pain 153: 1167–1179.
3. Tzaan WC, Chen HC (2011) Investigating the possibility of intervertebral disc regeneration induced by granulocyte colony stimulating factor-stimulated stem cells in rats. Adv Orthipol 2011: 602089.
4. Inoue N, Espinoria Oarias AA (2011) Biomechanics of intervertebral disk degeneration. Orthop Clin North Am 42: 487–499, vii.
5. Johnson WE, Eisenstein SM, Roberts S (2001) Cell cluster formation in degenerate lumbar intervertebral discs is associated with increased disc cell proliferation. Connect Tissue Res 42: 197–207.
6. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, et al. (1994) Positional cloning of the mouse obese gene and its human homologue. Nature 372: 425–432.
7. Sobhani I, Bado A, Visuzaine C, Bayse M, Kermorgant S, et al. (2009) Leptin secretion and leptin receptor in the human stomach. Gut 47: 178-183.
8. Hui W, Litherland GJ, Elias MS, Kitson GI, Caswton TE, et al. (2012) Leptin produced by joint white adipose tissue induces cartilage degradation via upregulation and activation of matrix metalloproteinases. Am Rheum Dis 71: 455-462.
9. Conidire RV, Sinha MK, Heinman ML, Kriauciunas A, Stephens TW, et al. (1996) Serum immunoreactive-leptin concentrations in normal-weight and obese humans. N Engl J Med 334: 292-295.
10. Mantzoros CS, Magkos F, Brinkoetter M, Sienkiewicz E, Dardeno TA, et al. (2011) Leptin in human physiology and pathophysiology. Am J Physiol Endocrinol Metab 301: E567-304.
11. Xu P, Ye W, Li H, Liu S, Kuo CT, et al. (2010) Zeranol enhances leptin-induced proliferation in primary cultured human breast cancer epithelial cells. Mol Med Report 3: 795-800.
12. Houss D, Housova J, Vernerova Z, Haluzik M (2006) Adipocytokines and cancer. Physiol Res 55: 233-244.
13. Gruber HE, Ingram JA, Hoelscher GL, Hanley EN Jr (2007) Leptin expression by annulus cells in the human intervertebral disc. Spine J 7: 437-443.
14. Zhao CQ, Liu D, Li H, Jiang LS, Dai LY (2008) Expression of leptin and its functional receptor on disc cells: contribution to cell proliferation. Spine (Phila Pa 1976) 33: E558-864.
15. Frühbeck G (2006) Intracellular signalling pathways activated by leptin. Biochem J 393: 7-20.
16. Cottrell EG, Mercer JG (2012) Leptin receptors. Handb Exp Pharmacol: 3-21.
17. Lam QL, Lu L (2007) Role of leptin in immunity. Cell Mol Immunol 4: 1-13.
18. Pai R, Lin C, Tran T, Tarnawski A (2005) Leptin activates STAT and ERK2 pathways and induces gastric cancer cell proliferation. Biochem Biophys Res Commun 331: 984-992.
19. Samartzis D, Karppinen J, Mok F, Fong DY, Luk KD, et al. (2011) A population-based study of juvenile disc degeneration and its association with overweight and obesity, low back pain, and diminished functional status. J Bone Joint Surg Am 93: 662-670.
20. Dahia CL, Mahoney E, Wylie C (2012) Shh signaling from the nucleus pulposus of the human intervertebral disc. J Orthop Res 30: 1470-1477.
21. Minogue BM, Richardson SM, Zeef LA, Freemont AJ, Hoyland JA (2010) Characterization of the human nucleus pulposus cell phenotype and evaluation of novel marker gene expression to define adult stem cell differentiation. Arthritis Rheum 62: 3695-3705.
22. Shimura T (2011) Acquired radioresistance of cancer and the AKT/GSK3beta/cyclin D1 overexpression cycle. J Radiat Res 52: 539-544.
23. Saxena NK, Vertino PM, Anania FA, Sharma D (2007) leptin-induced growth stimulation of breast cancer cells involves recruitment of histone acetyltransferases and mediator complex to CYCLIN D1 promoter via activation of Stat3. J Biol Chem 282: 13316-13325.
24. Catalano S, Giordano C, Rizza P, Gu G, Barone I, et al. (2009) Evidence that leptin through STAT and CREB signaling enhances cyclin D1 expression and promotes human endometrial cancer proliferation. J Cell Physiol 218: 490-500.
25. Chen C, Chang YC, Liu CL, Liu TP, Chang KF, et al. (2007) Leptin induces proliferation and anti-apoptosis in human hepatocarcinoma cells by up-regulating cyclin D1 and down-regulating Bax via a Janus kinase 2-linked pathway. Endoer Relat Cancer 14: 513-529.
26. Cremer BA, Sakamoto K, Schmidt JW, Triplett AA, Morinig R, et al. (2010) Stat3 promotes survival of mammary epithelial cells through transcriptional activation of a distinct promoter in Akt1. Mol Cell Biol 30: 2957-2970.
27. Prassina H, Kienas D (2007) PDGF, bFGF and IGF-I stimulation of the proliferation of intervertebral disc cells in vitro via the activation of the ERK and Akt signaling pathways. Eur Spine J 16: 1858-1866.
28. Gorka E, Popik K, Stehmaszek-Emmel A, Ciepila O, Kucharska A, et al. (2010) Leptin receptors. Eur J Med Res 15 Suppl 2: 30-54.
29. Simopoulos T, Malinos KN, Bieposinos D, Stefanou N, Papahodeou L, et al. (2007) Differential expression of leptin and leptin’s receptor isoform (Ob-Rb) mRNA between advanced and minimally affected osteoarthritic cartilage; effect on cartilage metabolism. Osteoarthr Cartil 15: 872-883.
30. Cheng SP, Yin PH, Hsu YC, Chang YC, Huang SY, et al. (2011) Leptin enhances migration of human papillary thyroid cancer cells through the PI3K/AKT and MEK/ERK signaling pathways. Oncol Rep 26: 1265-1271.
31. Trinko R, Gan G, Gao XB, Gu G, Barone I, et al. (2011) Erk1/2 mediates leptin receptor signaling in the ventral tegmental area. PLoS One 6: e27180.
32. Yin N, Wang D, Zhang H, Yi X, Sun X, et al. (2004) Molecular mechanisms involved in the growth stimulation of breast cancer cells by leptin. Cancer Res 64: 3870-3875.
33. Trout JJ, Backsalter JA, Moore KC (1982) Ultrastructure of the human intervertebral disc: II. Cells of the nucleus pulposus. Anat Rec 204: 307-314.
34. Roughead PJ (2004) Biology of intervertebral disc aging and degeneration: involvement of the extracellular matrix. Spine (Phila Pa 1976) 29: 2691-2699.
35. Studer RK, Zos N, Sowa G, Onudem K, Kang J (2011) Human nucleus pulposus cells react to IL-6: independent actions and amplification of response to IL-1 and TNF-alpha. Spine (Phila Pa 1976) 36: 593-599.
36. Navone SE, Marfia G, Canzi L, Guarnieri D, Canazza A, et al. (2012) Expression of neural and neurotrophic markers in nucleus pulposus cells isolated from degenerated intervertebral disc. J Orthop Res 30: 1470-1477.
37. Wang F, Yu J, Yang GH, Wang XS, Zhang JW (2011) Regulation of erythroid differentiation by miR-376a and its targets. Cell Res 21: 1196-1209.