Osteoarthritis and Cartilage

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Leptin receptor JAK2/STAT3 signaling modulates expression of Frizzled receptors in articular chondrocytes

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SUMMARY

Objective: Differentiated articular chondrocytes express a functional bisoform of the leptin receptor (LRb); however, leptin-LRb signaling in these cells is poorly understood. We hypothesized that leptin-LRb signaling in articular chondrocytes functions to modulate canonical Wnt signaling events by altering the expression of Frizzled (FZD) receptors.

Methods: Human chondrocyte cell lines and primary articular chondrocytes were grown in serum containing growth media for 24 h, followed by a media change to Dulbecco's modified Eagle's medium (DMEM) containing 1% Nutridoma-SP to obtain a serum-deficient environment for 24 h before treatment. Treatments included recombinant human leptin (10—100 nM), recombinant human IL-6 (0.3—3 nM), or recombinant human erythropoietin (Epo) (10 mU/ml). Cells were harvested 30 min—48 h after treatment and whole cell lysates were analyzed using immunoblots or luciferase assays.

Results: Treatment of cells with leptin resulted in activation of Janus kinase 2 (JAK2) and subsequent phosphorylation of specific tyrosine residues on LRb, followed by dose- and time-dependent increases in the expression of Frizzled-1 (FZD1) and Frizzled-7 (FZD7). Leptin-mediated increases in the expression of FZD1 were blocked by pre-treatment with the protein synthesis inhibitor cycloheximide or the JAK2 inhibitor AG490. Experiments using a series of hybrid Epo extracellular domain-leptin intracellular domain receptors (ELR) harboring mutations of specific tyrosine residues in the cytoplasmic tail showed that increases in the expression of FZD1 were dependent on LRb-mediated phosphorylation of STAT3, but not ERK1/2 or STAT5. Leptin pre-treatment of chondrocytes prior to Wnt3a stimulation resulted in an increased magnitude of canonical Wnt signaling.

Conclusion: These experiments show that leptin-LRb signaling in articular chondrocytes modulates expression of canonical Wnt signaling receptors and suggests that direct cross-talk between these pathways is important in determining chondrocyte homeostasis.

Introduction

Obesity is a major epidemiological risk factor for the development of knee osteoarthritis (OA), but molecular links between obesity and the risk of knee OA are unknown[1—6]. Over the last few years several laboratories have established hypothetical links between the adipokine leptin and development of knee OA[7—10]. Recently, it has been reported that leptin is significantly elevated in synovial fluid of patients with OA, providing an additional epidemiological link to the development of OA[7,11].

Leptin is a 16 kDa protein secreted by white adipose tissue, and encoded by the obese (ob/ob) gene[12]. Leptin signals via leptin receptors (LR), members of the class I cytokine receptor family[13] that include the prototypic interleukin 6 receptor (IL-6R). Six isoforms of LR have been identified, but the b isoform (LRb) is responsible for intracellular signal transduction[14,15]. LRb associates with Janus kinase 2 (JAK2) tyrosine kinase to initiate intercellular signaling via phosphorylation of LR tyrosine residues Tyr985, Tyr1077 and Tyr1138. Tyr985 and Tyr1138 recruit SH2 domain-containing tyrosine phosphatase (SHP-2) and signal transducer and activator of transcription-3 (STAT3), respectively[16]. Tyr985/SHP-2 is a major regulator of downstream extracellular signal-regulated kinase 1/2 (ERK1/2) activation, while Tyr1138/STAT3 induces expression of several downstream effectors including the negative feedback regulator SOCS3[17,18].

Leptin controls energy balance and body weight through LR signaling in the central nervous system (CNS), and regulates

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developmental and homeostatic aspects of osteoblast proliferation, collagen synthesis, bone growth and mineralization[19–23]. However, it remains unclear whether these effects are mediated via peripheral LR signaling events or exclusively by CNS pathways[22,23].

Wnts are members of a gene family representing mammalian orthologs of wingless, a *Drosophila* patterning gene (including 19 human Wnt genes). Wnt genes encode secreted glycoproteins that regulate critical aspects of morphogenesis in an extensive array of tissues[24]. Wnt-regulated signal transduction involves two well-described pathways, canonical signaling with activation of β-catenin and non-canonical signaling with intracellular calcium release and activation of protein kinase C[25]. Activation of both pathways is associated with binding of Wnts to their cognate receptors, a family known as Frizzled (FZD). In addition, Wnt/β-catenin signaling regulates expression of cyclo-oxygenase-2 and several matrix metalloproteinases (MMPs)[26–33]. MMP-13 has been shown to degrade arthritis cartilage in human joints[34]. Both human genetic data and transgenic mouse studies have linked the Wnt signaling antagonist soluble FZD-related protein 3 to protection against OA. Functional parallels between FZD and LRb signaling in chondrocytes and osteoblasts are worth noting as activation of either pathway yields significant effects on cell differentiation and composition of extracellular matrix[14,15]. A previous study showed that leptin deficiency protects animals from liver fibrosis, suggesting that leptin may modulate expression of metalloproteinases by target cells[13]. Collectively, these findings suggest the hypothesis that a mechanistic link between FZD and LRb signaling pathways functions in articular chondrocytes.

We show that LR signaling pathways are operative in articular chondrocytes and identify FZD receptors as downstream target genes. Although 10 human FZD receptors have been identified and characterized, in these initial studies we chose to examine a subgroup (FZD1, FZD2 and FZD7) that show similar structural organization at the genomic level and a high degree of shared amino acid identity (75%)[35]. We show that leptin stimulation induces expression of FZD1 and FZD7, but not FZD2. LRb activation resulted in JAK2, ERK1/2 and STAT3 phosphorylation. To identify specific LRb signaling pathways, an Epo-LR (ELR) chimera containing the extracellular domain of Epo receptor and the intracellular domain of leptin was used, since articular chondrocytes do not express Epo receptor. Mutation of Tyr1138, but not Tyr985 or Tyr1077, in the chimeric ELR blocked both STAT3 phosphorylation and leptin-induced FZD1 expression. We also demonstrate that leptin-mediated increases in the expression of FZD are associated with an increased magnitude of canonical Wnt signaling following exposure to recombinant Wnt3a. These results suggest "cross-talk" between leptin and Wnt signaling occurs in articular chondrocytes whereby leptin modulates expression of Wnt receptors through a JAK2/STAT3-dependent pathway.

Methods

Cell culture and cell stimulation

Human chondrocyte cell lines (C-28/I2 and T/C-28a2; Mary Goldring, Ph.D., Hospital for Special Surgery), an osteoblast cell line (hFOB1.19; ATCC), and human primary chondrocytes derived from cartilage of a 77-year-old female (Asterand, Detroit, MI) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 25 mM glucose (Invitrogen), 100 units/ml penicillin, 100 mg/ml streptomycin (Invitrogen), and 10% fetal bovine serum (Invitrogen) at 37 °C in 5% CO_2. Cells were seeded onto 10 cm dishes (5×10^5 cells) for 24 h in complete media, followed by DMEM 1% Nutridoma-SP (Roche) for 24 h before treatment with human recombinant leptin (Sigma, 100 nM unless otherwise noted), IL-6

(R&D Systems, 3 nM), or recombinant Epo (Amgen, 10 mU/ml). Where indicated, recombinant Dkk-1 (R&D Systems, 100 ng/ml) was added contemporaneously with leptin. Recombinant human Wnt3a (R&D Systems, 100 ng/ml) was added 6 h prior to cell harvest. Cycloheximide (Sigma, 10 mg/ml), JAK2 phosphorylation inhibitor Tyrphostin AG490 (LC Laboratories, 50 nM) and phosphoinositide 3-kinase (PI3K) inhibitors LY294002 (Calbiochem, 25 μM) and wortmannin (Sigma, 50 nM) were added 30 min before leptin.

Plasmids and plasmid transfection

Wild-type and tyrosine mutant ELR expression plasmids (Martin Myers, M.D., Ph.D., University of Michigan) are previously described[16]. pLentilox-topflash-3xTCF-LUC and pLentilox-reverse topflash-3xTCF-LUC were constructed by removing U6-shRNA and CMV-GFP expression cassettes from pLentiLox3.7 (Vector Core, University of Michigan), then inserting a topflash-3x TCF-Luciferase expression cassette (pLentilox-topflash-3xTCF-LUC). C-28/I2 and T/C-28a2 cells were passed 1 day prior to transfection on 10 cm plates (3×10^5 cells) in complete media. Cells were transfected using 2 μg of ELR plasmid and 8 μg polyethylenimine (PEI) in 400 μl Optimem I (no serum) (Invitrogen) for 20 min at room temperature, incubated an additional 24 h in complete media, and stimulated as described above.

Lentivirus production and transduction

Lentivirus packaging vectors (pMDL-RRE, pRSV-REV, pC1-VSVG) were co-transfected with pLentilox-topflash-3xTCF-LUC proviral plasmid into 293 T cells by the University of Michigan Vector Core using PEI precipitation. Supernatants were collected after 72 h, pelleted, then resuspended at 10X the original concentration ($\sim 1 \times 10^7$ TU/ml). C-28/I2 cells were seeded on a six well plate (1.5×10^5 cells/well) one day prior to lentiviral infection. Cell media was changed to 1.2 ml of fresh complete media, then 0.3 ml of 10x virus and 8 μg Polybrene (Sigma) were added and centrifuged at 2,500 rpm for 30 min at 27 °C.

Protein extraction and Western blotting

Cells were rinsed with ice-cold phosphate-buffered saline (PBS) and harvested in 200 μl RIPA buffer containing Complete, Mini protease inhibitor cocktail (Roche) and 1X Halt phosphatase inhibitor cocktail (Pierce). Equal amounts of lysate were subjected to SDS-PAGE using 10% Ready-Gel polyacrylamide gels (Bio-Rad Laboratories) and transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) as described[36]. Membranes were blocked in PBS containing 0.1% tween-20 (PBST) plus 5% nonfat dry milk for 1 h before incubation with primary antibody (anti-JAK2, anti-phosphorylated JAK2 (pJAK2), anti-ERK, anti-phosphorylated ERK3 (pERK3), anti-STAT3, anti-phosphorylated STAT3 (pSTAT3), anti-Akt, anti-phosphorylated Akt (pAkt), anti-glyceraldehyde 3 phosphate dehydrogenase (GAPDH) (Cell Signaling), anti-Bone Morphogenic Protein Receptor 1A (BMPR-1A, Zymed), anti-FZD1 (R&D), anti-FZD2 (Abcam) and anti-FZD7 (Abcam)) overnight at 4 °C. All primary antibodies were diluted 1:1000 except STAT3 (1:2000) and GAPDH (1:4000) in PBST plus 5% nonfat dry milk. After washing with PBST, membranes were incubated with horse-radish peroxidase-conjugated donkey anti-rabbit secondary antibodies (Cell Signaling) diluted 1:2000 in PBST plus 5% nonfat dry milk for 1 hr at room temperature. After washing with PBST, immunoreactive bands were visualized using chemoluminescence reagents (Pierce) and exposed to film (CL-Xposure Film, Thermo Scientific). Probed blots were stripped (25 mM glycine, 1% SDS, pH

2.0) for 5 min at room temperature and re-probed for GAPDH for normalization. Densitometry was calculated using Scion Image software, and values expressed as mean + 95% confidence interval with statistical significance determined by Student's *t*-test.

Luciferase assay

Cells transduced with Lentivirus-topflash-3xTCF-LUC were cultured as described above. Cells were harvested in 1x Reporter Lysis Buffer (Promega) and lysates were centrifuged and assayed for luciferase activity (Luciferase Assay System, Promega) and quantified (Monolight 3010 luminometer, BD Biosciences). Whole cell lysates were assayed in triplicate and results expressed as relative light units (RLU) normalized to milligrams of total protein content (Bradford Reagent, Bio-Rad). Statistical significance was evaluated by Student's *t*-test.

Results

Leptin induces expression of FZD1 and FZD7 in articular chondrocytes

We examined whether leptin-LRb signaling was a regulator of Wnt signaling via FZD receptors in human articular chondrocytes (C-28/I2 and T/C-28a2 cell lines). These cells express type II collagen, decorin, aggrecan and other proteins associated with a differentiated articular chondrocyte phenotype, and have been used extensively as *in vitro* model systems for studies of chondrocyte biology[37,38]. C-28/I2 cells were treated with leptin (100 nM) and harvested at 0, 6, 12, 24 and 48 h after leptin treatment, and FZD receptor protein levels determined. Leptin stimulation resulted in a significant increase in FZD1 and FZD7 protein levels as early as 24 h after treatment and a 3-fold increase within 48 h of treatment [Fig. 1(A) and (B)]. Leptin stimulation had little effect on FZD2 protein levels in the C-28/I2 cells [Fig. 1(B)]. Leptin stimulation of the T/C-28a2 cell line also showed induction of FZD1 similar to C-28/I2 cells, but not FZD7 or FZD2 (data not shown) while GAPDH showed no significant change between samples.

To further explore the mechanism of leptin-induced FZD1 increases, we added cycloheximide 30 min prior to leptin treatment [Fig. 1(C)]. As expected, leptin-induced FZD1 expression was significantly blocked in the presence of cycloheximide, while GAPDH showed no significant change in levels before and after treatment with leptin. These results are consistent with leptin-induced *de novo* protein synthesis of FZD1.

Leptin induces JAK2, ERK1/2 and STAT3 phosphorylation in articular chondrocytes

It has been shown, primarily in the CNS, that leptin binding to LRb activates JAK2 phosphorylation, which, in turn, activates ERK1/2 and STAT3 signaling cascades. To test whether leptin functions through a similar signaling cascade in articular chondrocytes, C-28/I2 cells were treated with leptin and analyzed in a time-dependent manner. Whole cell extracts were examined for both phosphorylated and non-phosphorylated JAK2 [Fig. 2(A)]. Leptin treatment caused increased JAK2 phosphorylation within 10 min and increased levels of phosphorylated JAK2 were observed for approximately 30 min. Densitometry showed a 1.7- and 1.5-fold increase in JAK2 stimulation at 10 and 30 min, respectively, with no significant change in total protein pools of JAK2 and GAPDH. Similar results were observed in articular chondrocyte cell line T/C-28a2 (data not shown).

To further define specific aspects of LRb signaling, ERK1/2 phosphorylation levels were analyzed in a time-dependent manner. Leptin significantly induced ERK1/2 phosphorylation as early as
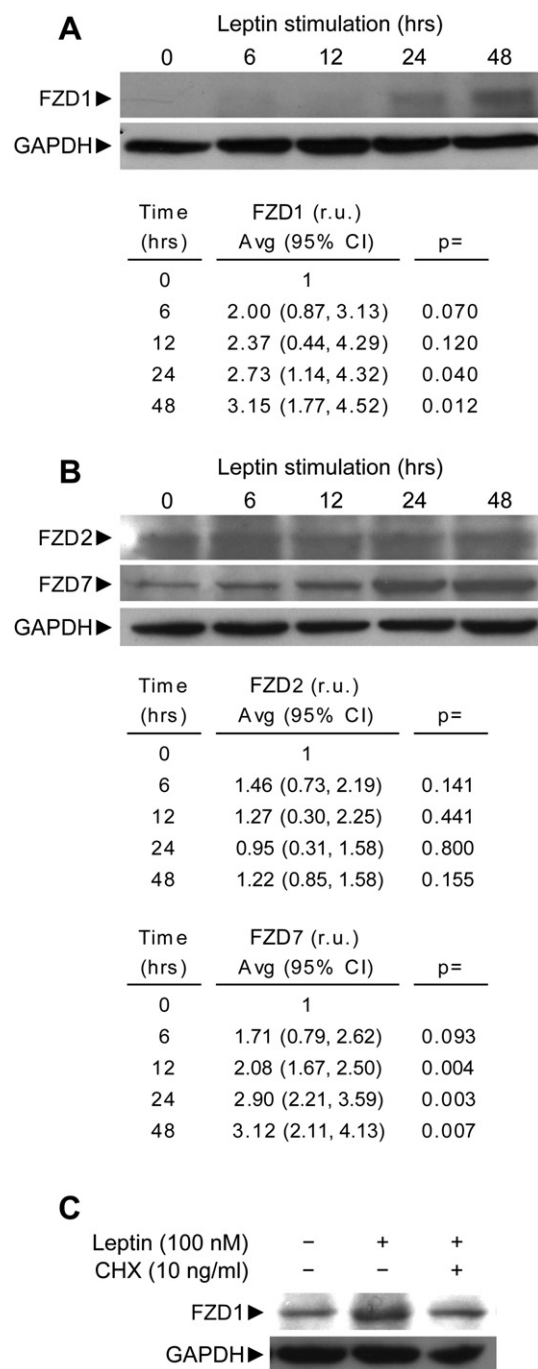

A

Leptin stimulation (hrs)

Time (hrs)	FZD1 (r.u.) Avg (95% CI)	p=
0	1	
6	2.00 (0.87, 3.13)	0.070
12	2.37 (0.44, 4.29)	0.120
24	2.73 (1.14, 4.32)	0.040
48	3.15 (1.77, 4.52)	0.012

B

Leptin stimulation (hrs)

Time (hrs)	FZD2 (r.u.) Avg (95% CI)	p=
0	1	
6	1.46 (0.73, 2.19)	0.141
12	1.27 (0.30, 2.25)	0.441
24	0.95 (0.31, 1.58)	0.800
48	1.22 (0.85, 1.58)	0.155

Time (hrs)	FZD7 (r.u.) Avg (95% CI)	p=
0	1	
6	1.71 (0.79, 2.62)	0.093
12	2.08 (1.67, 2.50)	0.004
24	2.90 (2.21, 3.59)	0.003
48	3.12 (2.11, 4.13)	0.007

C

Leptin (100 nM)   −   +   +
CHX (10 ng/ml)   −   −   +

Fig. 1. LR signaling induces expression of FZD1 in articular chondrocytes. Chondrocytes (C-28/I2) were treated with recombinant leptin (100 nM) and harvested over a time course (0–48 h). Whole cell lysates were separated by SDS-PAGE and analyzed using antibodies specific for (A) FZD1, (B) FZD2 and FZD7 protein. Band intensities (shown as representative photographs) were quantified using densitometry, and in each case *t* = 0 was set to 1 r.u. Tables represent means of four or more separate experiments with 95% confidence intervals represented as (lower limit, upper limit) and *P*-values. Significant increases in levels of FZD1 and FZD7 protein were observed at 24 and 48 h, but no change in levels of FZD2 was observed. (C) C-28/I2 cells pre-treated with cycloheximide (10 ng/ml) prior to treatment with recombinant leptin (100 nM) did not increase levels of FZD1 in response to leptin treatment. Whole cell lysates were separated by SDS-PAGE and analyzed using antibodies specific for FZD1. GAPDH protein levels analyzed as controls remained unchanged in all experiments.
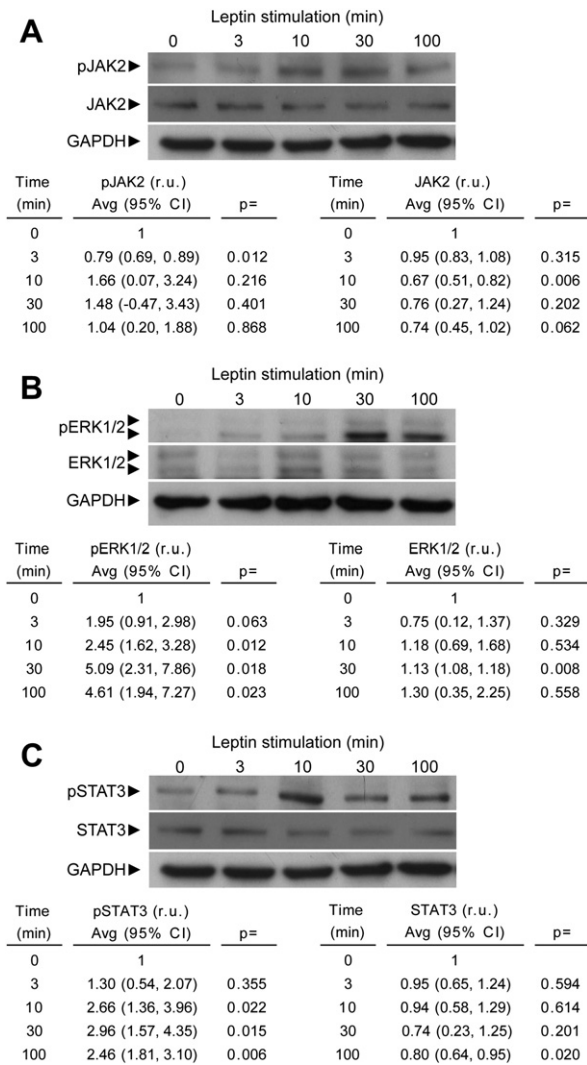
Fig. 2. Leptin stimulation induces phosphorylation of JAK2, ERK1/2 and STAT3 in articular chondrocytes.
Chondrocyte cell lines (C-28/I2 and T/C-28a2) were treated with recombinant leptin (100 nM) and harvested over a time course (0–100 min). (A) C-28/I2 whole cell lysates were separated by SDS-PAGE and analyzed using antibodies specific for JAK2 and pJAK2. (B) T/C-28a2 whole cell lysates were separated by SDS-PAGE and analyzed using antibodies specific for ERK1/2 and pERK1/2. (C) C-28/I2 whole cell lysates were separated by SDS-PAGE and analyzed using antibodies specific for STAT3 and pSTAT3. Band intensities (shown as representative photographs) were quantified using densitometry and in each case $t = 0$ was set to 1 r.u. Tables represent the mean of three or more separate experiments with 95% confidence intervals represented as (lower limit, upper limit) and P-values. Leptin treatment led to significant increases in pJAK2 at 10 and 30 min, pERK1/2 at 30 and 100 min, and pSTAT3 at 10, 30 and 100 min. GAPDH protein levels analyzed as controls remained unchanged in all experiments.

Fig. 3. Leptin stimulation induces phosphorylation of STAT3 and expression of FZD1 and FZD7 in primary articular chondrocytes.
Primary human articular chondrocytes (passage 2) were treated with leptin (100 nM) and harvested over a time course (0–48 h). Whole cell lysates were separated by SDS-PAGE and analyzed using antibodies specific for STAT3, phospho-STAT3, FZD1 and FZD7. Leptin treatment induces pSTAT3 accumulation at 30 min (A), increases in the levels of FZD1 (B) and FZD7 (C) at both 24 and 48 h. GAPDH protein levels analyzed as controls remained unchanged in all experiments.

10 min after treatment and increased levels were sustained for at least 100 min [Fig. 2(B)] in T/C-28a2 cells. A maximum 5-fold stimulation was observed at 30 min. As internal controls, total protein levels of ERK1/2 and GAPDH remained unchanged between samples. Unexpectedly, leptin stimulation of ERK1/2 was not observed in C-28/I2 cells. In order to reconcile the observed differences between C-28/I2 and T/C-28a2 cell lines, we repeated the leptin stimulation experiments using primary human chondrocytes. These studies showed that leptin-LRb signaling did not result in significant time-dependent phosphorylation of ERK1/2 (data not shown). In summary, these results suggest that leptin-LRb activation of ERK1/2 is not likely to be a major downstream signaling pathway that leads to induced expression of FZD1 in articular chondrocytes.
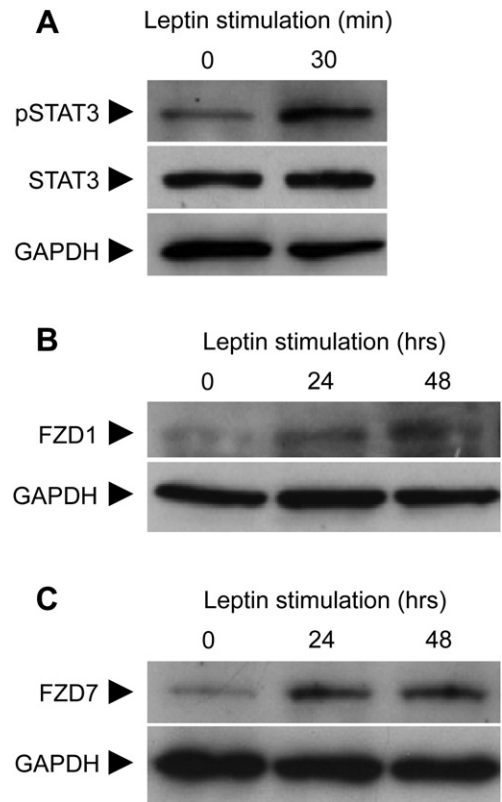
To test whether leptin stimulation activates time-dependent STAT3 phosphorylation, the C-28/I2 cell line was treated with increasing concentrations of leptin and examined for both phosphorylated and non-phosphorylated STAT3. Leptin caused increased STAT3 phosphorylation at 62 nM and 1 μM concentrations as early as 5 min after stimulation and increases were observed for at least 30 min (data not shown), consistent with published reports in other leptin-responsive cell types[39]. Rapid STAT3 phosphorylation suggests direct activation from the stimulated LRb receptor. Based on these results, a leptin concentration of 100 nM was used for all subsequent experiments.

To quantify leptin-induced STAT3 phosphorylation, an extended time course was performed [Fig. 2(C)]. Leptin induced STAT3 phosphorylation within 10 min and levels remained elevated 100 min post-treatment. Densitometry indicated a 2.7- and 3.0-fold increase in STAT3 stimulation at 10 and 30 min, respectively, following leptin treatment. As controls, total protein pools of STAT3 and GAPDH were determined and showed no significant change between samples. Similar results were observed in T/C-28a2 cells (data not shown).

Leptin induces STAT3 signaling and FZD1 expression in human primary chondrocytes

To show that leptin effects were not specific to immortalized chondrocyte cell lines, identical experiments were performed using primary human chondrocytes [Fig. 3]. Treatment with 100 nM leptin resulted in robust phosphorylation of STAT3 [Fig. 3(A)], while

total protein levels of STAT3 and GAPDH showed no change. Primary chondrocytes were treated with 100 nM leptin for 24 and 48 h prior to harvest and analyzed for expression of FZD1 and FZD7 [Fig. 3(B) and (C)]. Similar to the C-28/I2 cell line, significant increases in FZD1 and FZD7 protein levels were observed at 24 and 48 h after leptin treatment, while GAPDH remained unchanged.

FZD1 expression in chondrocytes is not regulated by IL-6

Leptin and IL-6, both members of the IL-6 cytokine superfamily, activate closely related receptors. LRb and IL-6R have been shown to activate similar JAK2, ERK1/2 and STAT3 signal transduction pathways[24,25]. Furthermore, IL-6 is also secreted by adipose cells, causes major effects on cells outside of the CNS and has been implicated in several forms of arthritis. Therefore, as a control we compared the function of IL-6 signaling with respect to regulation of FZD1 expression in articular chondrocytes. To assess IL-6R signaling, C-28/I2 cells were treated with IL-6 and cells were harvested at various time points. We detected no increase in FZD1 expression at 6, 12, 24, or 48 h after IL-6 treatment [Supplementary Fig. 1(A)]. Surprisingly, IL-6 stimulation of chondrocytes did not significantly induce phosphorylation of JAK2, ERK1/2, or STAT3 at all time points up to 100 min [Supplementary Fig. 1(B)–(D)]. Total protein pools of JAK2, ERK1/2, STAT3 and GAPDH remained unchanged in these studies. As a positive control, IL-6 treatment of C-28/I2 cells resulted in a 2-fold increase in the expression of BMPR-1A [Supplementary Fig. 1(E)], as previously reported[40]. Collectively, our data suggest that LRb and IL-6R act through distinct downstream signaling pathways in articular chondrocytes. The results indicate that the observed induction of FZD1 in articular chondrocytes is a specific effect of leptin treatment.

Leptin induces STAT3 signaling and FZD1 expression in osteoblasts

OA is a complex disease that affects both chondrocytes and osteoblasts in humans. To determine whether leptin affects modulation of Wnt signaling in both chondrocytes and osteoblasts through a conserved mechanism, we investigated whether leptin-LRb-induced FZD1 expression was also operative in human osteoblasts. hFOB1.19 cells were treated with leptin (0.62 to >620 nM) for 15 and 30 min before cells were harvested and analyzed for STAT3 phosphorylation [Fig. 4(A)]. Similar to observations in chondrocytes, dose-responsive phosphorylation of STAT3 was observed at both the 15 and 30-min time points, while the total protein pools of STAT3 and GAPDH remained relatively unchanged. Additional studies were then performed in hFOB1.19 cells to analyze FZD1, FZD2 and FZD7 expression following leptin treatment. The hFOB1.19 cells were treated with 100 nM leptin for 0, 6, 12, 24, and 48 h prior to harvest and analyzed. Similar to chondrocytes, significant increases in FZD1 protein levels were observed at 24 and 48 h after leptin treatment. Interestingly, FZD7 and FZD2 protein levels remained relatively unchanged [Fig. 4(B)], results similar to those observed in the T/C-28a2 chondrocyte cell line. These data suggest that leptin-LRb signaling-induced FZD1 expression may be operative in both articular chondrocytes and osteoblasts.

Leptin-induced JAK2-STAT3 phosphorylation and FZD1 expression depends on LRb receptor stimulation

To test whether the leptin-activated LRb-JAK2 signaling cascade was required for FZD1 induction, the JAK2 phosphorylation inhibitor AG490 was added to C-28/I2 cells 30 min prior to leptin treatment. 48 h after leptin treatment, cells were harvested and examined for FZD1 [Fig. 5(A)]. AG490 blocked leptin-
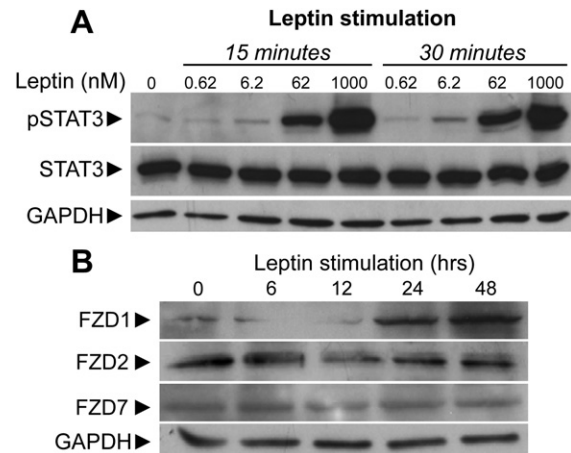

Fig. 4. Leptin stimulation induces phosphorylation of STAT3 and expression of FZD1 in osteoblasts.
Human fetal osteoblast cells (hFOB1.19) were treated with increasing concentrations of leptin (0.62 to >620 nM) and harvested over a time course (0–30 min or 0–48 h). Whole cell lysates were separated by SDS-PAGE and analyzed using antibodies specific for STAT3, phospho-STAT3, FZD1, FZD2 and FZD7. (A) Dose-dependent activation of pSTAT3 was observed at both 15 and 30 min. (B) Leptin treatment (100 nM) resulted in increased expression of FZD1, but no change in the levels of FZD2 and FZD7 expression. Bands are shown as representative photographs from three or more separate experiments. GAPDH protein levels analyzed as controls remained unchanged in all experiments.

stimulated expression of FZD1 induction, while GAPDH protein levels were unaffected. These results suggest that the leptin-activated LRb-JAK2 signaling cascade is necessary for induced expression of FZD1.

Once activated by the LRb receptor, phosphorylated JAK2 activates multiple downstream signaling pathways, including PI3K [Fig. 6(A)]. To examine whether the JAK2-activated PI3K pathway was required for FZD1 induction, PI3K inhibitors LY294002 and wortmannin were added to C-28/I2 cells 30 min before leptin treatment. 48 h after leptin treatment cells were harvested and examined for FZD1 protein levels [Fig. 5(B) and (C)]. These results showed that PI3K inhibitors had no effect on basal FZD1 levels or leptin-induced FZD1 expression. As a control to show that leptin-induced PI3K activity could be inhibited in these cells, we determined levels of phosphorylated Akt (a known downstream target of JAK2-activated PI3K signaling) following leptin stimulation [Fig. 6(D)]. Leptin stimulation of C-28/I2 cells resulted in a strong increase in phosphorylated Akt 30 min after stimulation. Addition of PI3K inhibitors LY294002 and wortmannin 30 min before leptin treatment inhibited leptin-induced phosphorylation of Akt. Total protein levels of both Akt and GAPDH showed little change with addition of leptin or the inhibitors, suggesting that the LRb-JAK2-PI3K pathway is not necessary for FZD1 induction.

To investigate the role of LRb-mediated JAK2-STAT phosphorylation on induced FZD expression, we used an Epo-leptin chimeric receptor (ELR), as articular chondrocytes do not express the Epo receptor [Fig. 6(A)]. ELR is a fusion protein containing the extracellular domain of the Epo receptor and an intracellular domain of LRb. C-28/I2 cells were transfected in the presence or absence of plasmid expressing wild-type ELR protein and subjected to treatment with 10 mU/ml Epo. Cells were harvested 30 min after Epo treatment and analyzed for STAT3 phosphorylation [Fig. 6(B)]. There was no increase in STAT3 phosphorylation upon Epo treatment in the absence of ELR, or with the expression of ELR in the absence of Epo; however, there was a significant increase in STAT3 phosphorylation with Epo treatment in ELR
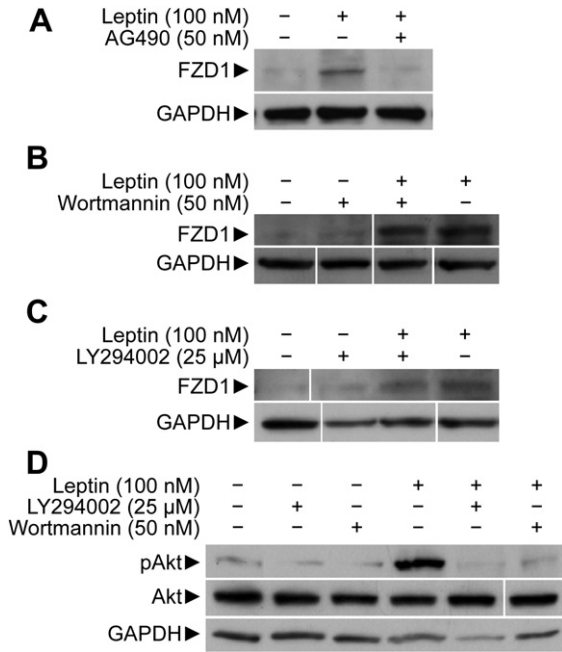
Fig. 5. Leptin-mediated FZD1 expression is dependent on LRb-JAK2 function but not PI3K/Akt activity.
C-28/I2 cells were pre-treated with (A) the JAK2 inhibitor AG490 (50 nM), (B) the PI3K inhibitor LY294002 (25 uM), and (C) the PI3K inhibitor wortmannin (50 nM) followed by treatment with leptin (100 nM). Cells were harvested at 24 h and whole cell lysates were separated by SDS-PAGE and analyzed using antibodies specific for FZD1. Leptin-mediated increases in the expression of FZD1 were inhibited by pre-treatment with AG490 but unchanged by pre-treatment with LY294002 or wortmannin. (D) Replicates were harvested 30 min post-leptin treatment and analyzed using Akt and pAkt-specific antibodies. Leptin-mediated activation of phospho-Akt was inhibited by pre-treatment with either LY294002 or wortmannin. Bands are shown as representative photographs from four or more separate experiments. White lines separate nonadjacent lanes from the same gel. GAPDH protein levels analyzed as controls remained unchanged in all experiments.

transfectants. GAPDH levels remained constant between samples throughout these experiments. We also used ELR transfectants to determine whether leptin-induced FZD1 expression was due to tyrosine phosphorylation of LRb. FZD1 protein levels remained unchanged at 24 and 48 h with either Epo treatment alone or ELR alone [Fig. 6(C)], and were significantly higher only in the presence of both ELR and Epo at both 24 and 48 h. GAPDH protein levels remained unchanged between samples throughout the experiments.

To test whether LRb-induced FZD1 expression is acting through phosphorylation of specific tyrosine residues, ELR constructs containing specific point mutations were used. ELR mutants included: ELR^{L985}, Tyr 985→leucine (Leu); ELR^{P1077}, Tyr 1077→phenylalanine (Phe); ELR^{S1138}, Tyr 1138→serine (Ser) [Fig. 7(A)]. Following treatment with Epo, increased phospho-STAT3 was observed in both ELR^{L985} and ELR^{P1077} mutant transfectants but not in an ELR^{S1138} mutant transfectant [Fig. 7(A)]. Experiments were repeated and analyzed for Epo-induced expression of FZD1 at 24 and 48 h. As expected, Epo treatment of both ELR^{L985} and ELR^{P1077} transfectants was associated with increased expression of FZD1, but no increase in FZD1 expression was observed following Epo treatment of ELR^{S1138} transfectants [Fig. 7(B)]. In all mutant ELR transfection experiments, GAPDH protein levels showed no significant differences between samples. These data indicate that in articular chondrocytes, the LRb-JAK2-STAT3 signaling cascade functions through LRb Tyr1138, but not Tyr985 or Tyr1077, and is required for leptin-mediated increases in the expression of FZD1.
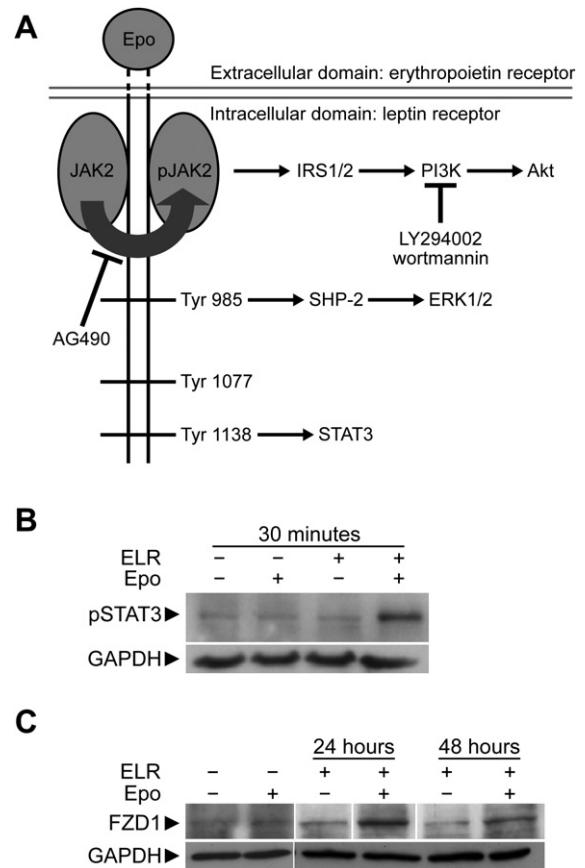

Fig. 6. LRb-mediated FZD1 expression is dependent on phosphorylation of tyrosine residues on LRb.
(A) Schematic representation of the hybrid receptor ELR consisting of the extracellular domain of the Epo receptor and the intracellular domain of LRb. Indicated are JAK2, JAK2-induced phosphorylated tyrosine residues (Tyr985, Tyr1077 and Tyr1138) and respective downstream signaling pathways. C-28/I2 cells were transfected with ELR expression plasmid (2 μg), treated with recombinant Epo (10 mU/ml) and harvested over a time course (0–48 h). Whole cell lysates were analyzed using antibodies specific for pSTAT3 (B) and FZD1 (C). Epo treatment of ELR transfectants induced phosphorylation of STAT3 (30 min) and increased expression of FZD1 at both 24 and 48 h. Bands are shown as representative photographs from three or more separate experiments. White lines separate nonadjacent lanes from the same gel. GAPDH protein levels analyzed as controls remained unchanged during Epo treatment.

Leptin modulates Wnt/β-catenin signaling in articular chondrocytes

To understand the functional consequence of leptin-LRb-induced expression of FZD, we derived transduced C-28/I2 cells that harbor a transgene consisting of three tandem Lef/TCF promoter elements upstream of the cDNA for firefly luciferase. Activation of canonical Wnt/β-catenin signaling via FZD/LRP5/6 receptor complexes in these transduced cells induces dose-dependent luciferase expression. Transduced C-28/I2 cells were treated with leptin alone (30 nM) for 24 h, Wnt3a alone (100 ng/ml) for 6 h, or sequentially with leptin for 24 h followed by Wnt3a for 6 h prior to harvest. Leptin treatment alone showed no significant increase in luciferase activity, while Wnt3a alone showed an approximately 2.5-fold increase in relative luciferase activity over untreated controls [Fig. 8]. Leptin treatment prior to Wnt3a stimulation increased relative luciferase activity approximately 6-fold. As a negative control, the soluble LRP6 inhibitor Dkk-1 was added to replicates for each condition and completely blocked Wnt3a-induced luciferase activity. These results show that leptin-LRb signaling induces expression of FZD and in turn increases responsiveness of the chondrocytes to Wnt/β-catenin signaling and target gene expression.
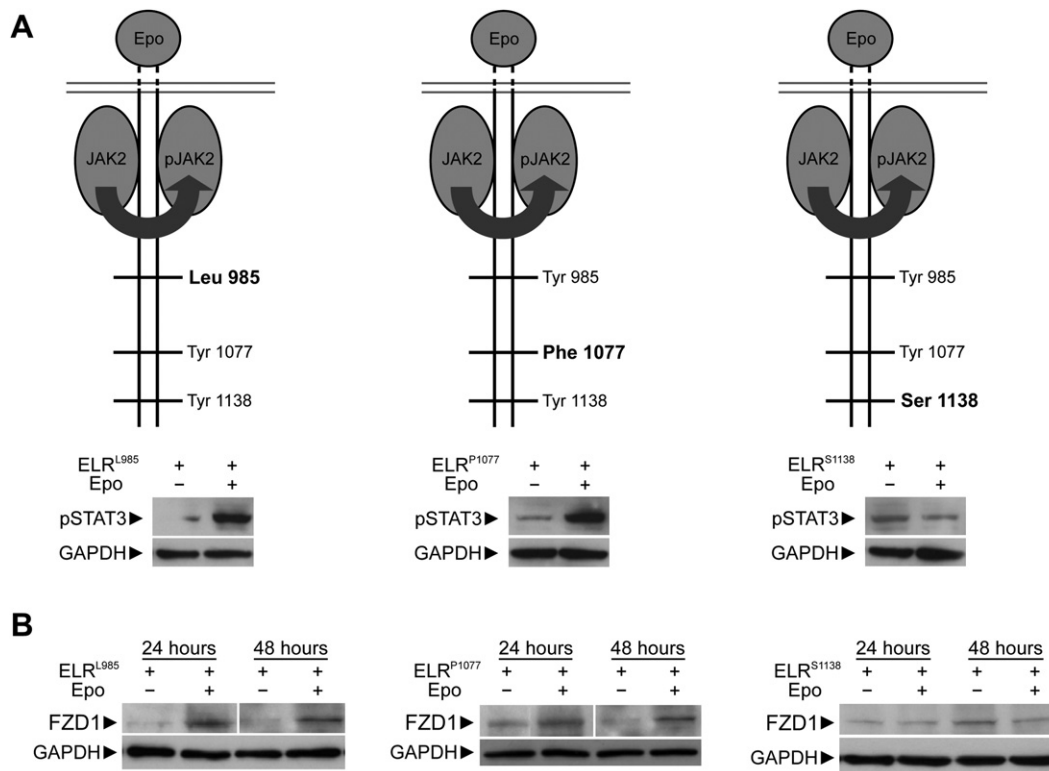
Fig. 7. LRb-mediated FZD1 expression is Tyr1138-pSTAT3 dependent.
ELR variants containing substitutions of critical tyrosine residues (Tyr-Leu965, Tyr-Phe1077 and Tyr-Ser1138) were constructed into expression plasmids. Plasmids for ELR[L985], ELR[P1077] and ELR[S1138] (2 μg) were transfected separately into C-28/I2 cells. Cells were treated with recombinant Epo (10 mU/ml) and harvested at 30 min or 24 h, and whole cell lysates were analyzed using antibodies specific for pSTAT3 and FZD1. (A) Epo-mediated ELR activation of pSTAT3 was unaffected in the ELR[L985] and ELR[P1077] transfectants but was blocked in ELR[S1138] transfectants. (B) Similarly, Epo-ELR-mediated increases in the expression of FZD1 were observed in ELR[L985] and ELR[P1077] transfectants but no increase in the expression of FZD1 was observed in the ELR[S1138] transfectants. Bands are shown as representative photographs from three or more separate experiments. White lines separate nonadjacent lanes from the same gel. GAPDH protein levels analyzed as controls remained unchanged during Epo treatment.

Discussion

Leptin controls energy balance and body weight through leptin-LRb signaling events that occur in specific CNS cells[17,41–45]. Although several studies have shown that functional LRb is expressed in both chondrocytes and osteoblasts, the physiological role of peripheral leptin-LRb signaling in the adult musculoskeletal system remains unknown[39,46,47]. Both acute and chronic exposure to leptin have shown that leptin-LRb signaling events have significant effects on osteoblasts, including regulation of proliferation, collagen synthesis, and bone mineralization; however, it is not clear whether these leptin effects are direct or indirect[23,47].

Studies in murine models of chondrocytes isolated from regions of endochondral ossification have shown that leptin-LRb signaling is associated with increases in proliferation rates, insulin-like growth factor-1 receptor abundance and type X collagen expression[48,49]. Leptin treatment also suppressed induction of apoptosis in the murine endochondral chondrocytes[48]. Similarly, leptin treatment of rabbit growth plate chondrocytes increased rates of proliferation and proteoglycan expression[50].

Although several investigators have shown that primary human articular chondrocytes express functional LRb, the physiological consequences of leptin-LRb signaling events in differentiated articular chondrocytes remain to be determined[8,51,52]. The study of Simopoulou and colleagues showed that long-term continuous exposure (5—7 days) of primary human chondrocytes to leptin led to increases in amounts of IL-1b, MMP-9 and MMP-13 proteins in treated cells[52]. However, these changes in protein levels were not directly tied to specific LRb signaling events and did not exclude the activation of other cell signaling pathways.

In this study we have shown that leptin-LRb signaling leads to increases in the expression of FZD1 and FZD7 in both human articular chondrocytes and osteoblasts. FZDs are seven-pass transmembrane proteins that act as receptors for their cognate glycoprotein ligands known as Wnts[35]. Binding of Wnts to FZD receptors leads to signaling events that activate both canonical and non-canonical downstream pathways. Canonical Wnt signaling leads to inhibition of β-catenin degradation, its accumulation and nuclear translocation. Nuclear β-catenin interacts with transcription factors such as lymphoid enhancer-binding factor-1/T cell-specific transcription factor to affect transcription of specific Wnt target genes. The canonical Wnt/β-catenin signaling pathway plays a central role in a variety of developmental processes including primary musculoskeletal morphogenesis, cell differentiation and proliferation, and tissue remodeling[53]. Wnt signaling has been shown to regulate multiple physiological pathways in both osteoblasts and chondrocytes to elicit matrix catabolism[54–59].

The effects of canonical Wnt/β-catenin signaling pathways in differentiated articular chondrocytes in adult animals are less clearly defined. However, some evidence suggests that Wnt-β-catenin activation may regulate expression of enzymes involved in degradation of extracellular matrix. Several reports have linked canonical Wnt/β-catenin signaling to transcriptional regulation of cyclo-oxygenase-2 expression in cancer cells, and another study has associated Lef1 with regulation of cyclo-oxygenase-2 expression in chondrocytes[27,60–62]. Activation of β-catenin in maturing chondrocytes is associated with
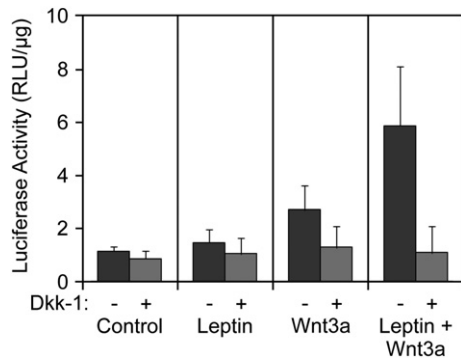
Fig. 8. Leptin modulates Wnt/β-catenin signaling in articular chondrocytes.
C-28/I2 cells were transduced with a recombinant lentivirus that contains three tandem Lef/TCF promoter elements upstream of the cDNA for firefly luciferase. Activation of canonical Wnt/β-catenin signaling induces luciferase expression in the transduced cells. Parallel aliquots of transduced cells were treated with recombinant human leptin (30 nM), recombinant human Wnt3a (100 ng/ml), or sequentially with leptin followed by Wnt3a. Recombinant human Dkk-1 (100 ng/ml) was added to replicates as a soluble Wnt antagonist. Whole cell lysates were assayed in triplicate for luciferase activity and the results expressed as RLU normalized to μg of total protein content. Leptin treatment did not significantly increase luciferase activity. Wnt3a treatment increased luciferase activity (approximately 2.5-fold over controls) and leptin plus Wnt3a treatment significantly increased activity compared to controls, leptin and Wnt3a treatment (approximately 6-fold over controls). In every case increases in luciferase activity were inhibited by treatment with Dkk-1. Columns indicate mean values with 95% confidence intervals determined from at least five separate experiments. Statistical significance was evaluated by Student's t-test. In comparing Wnt3a treatment to the control (Wnt3a vs Control), $P = 0.01$; Wnt3a vs Leptin, $P = 0.021$; Wnt3a vs Wnt3a+Dkk-1, $P = 0.008$; Leptin+Wnt3a vs Control, $P = 0.004$; Leptin+Wnt3a vs Leptin, $P = 0.005$; Leptin+Wnt3a vs Wnt3a, $P = 0.017$; and Leptin+Wnt3a vs Leptin+Wnt3a+Dkk-1, $P = 0.001$.

increased expression of multiple MMPs including MMP-3, MMP-9 and MMP-13[54]. Other studies performed in cancer cells have shown that canonical Wnt-β-catenin signaling can regulate the expression of MMP-9 and MMP-13[63,64]. Both dysregulated expression of MMPs and cyclo-oxygenase-2 have been implicated in the pathophysiology of OA[65,66].

Our data showed that leptin treatment of human chondrocytes and osteoblasts induced *de novo* expression of FZD1 and FZD7 in both a time- and dose-dependent manner [Fig. 1 and Fig. 4]. Significant increases in the expression of FZD1 and FZD7 protein occurred within 24 h following treatment with leptin. In addition, we have shown that leptin-mediated increases in FZD1 protein expression are not dependent on LRb-PI3K-Akt signaling but rather are LRb-JAK2-STAT3 signaling dependent. FZD receptors have been categorized into sub-families based on amino acid sequence and structure. Human FZD1, FZD2, and FZD7 share a common structure consisting of seven transmembrane domains, a cysteine-rich domain in the N-terminal extracellular region, and the C-terminal Ser/Thr-Xxx-Val motif, and share a high percentage of shared primary amino acid sequences (>75%)[67]. Interestingly, in our experiments, FZD2 expression was not increased in either chondrocytes or osteoblasts following leptin treatment. This observation suggests that transcriptional regulation of specific FZD family members likely includes cell type-specific factors that have yet to be identified.

Our data supports the hypothesis that leptin-LRb signaling regulates the expression of Wnt/β-catenin signaling pathway components in chondrocytes and osteoblasts and allows for direct cross-talk to occur between these two important signaling pathways. Additional investigation into the co-regulation of these important signaling pathways in chondrocytes and osteoblasts may provide insight into the pathophysiological changes that lead to the development of OA.

Author contributions

B.J.R. developed the hypotheses and directed all of the experiments. S.O. and T.M.L. conducted the experiments and constructed figures and text for the manuscript. All authors participated in experimental design of the study, statistical analysis and interpretation of data, revision of the manuscript, and final approval of the article.

Role of the funding source

Conflict of interest
The authors declare that no conflicts of interest exist.

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Supplementary data

Supplementary data associated with this article can be found in online version at doi:10.1016/j.joca.2010.09.006

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