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C/EBP-α, involvement of a novel transcription factor in leptin-induced VCAM-1 production in mouse chondrocytes

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ABSTRACT

Leptin and vascular cell adhesion molecules-1 (VCAM-1) are two important mediators in obesity-related osteoarthritis, while the molecular mechanism linking leptin to VCAM-1 production is still obscure. Here we show that leptin upregulates VCAM-1 mRNA and protein levels in a time- and dose-dependent manner. Mechanistically, leptin induces VCAM-1 promoter activity by increasing the expression of C/EBP-α and facilitating its binding to a newly identified element in the VCAM-1 gene. Gain or loss of function studies reveal a regulatory role of C/EBP-α on VCAM-1 expression. Finally, elevated plasma leptin level correlates to increased C/EBP-α and VCAM-1 production in chondrocytes from obese mice.

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1. Introduction

Osteoarthritis (OA), a most common rheumatic disease, is characterized by irreversible destruction of the joint cartilage. Biochemical, genetic and mechanical factors contribute to the onset and progress of this disorder. Lots of studies focus on the mechanical role of obesity on OA [1], while abnormal adipokines from dysfunctional adipose tissues are emerging as relevant factors which affect the cartilage structure [2,3].

Leptin, a most important adipose-derived hormone, is originally identified as a controller of food intake through its receptors [4]. It also has been reported that leptin signaling functions in chronic inflammation [5], insulin resistance [6], and tumorogenesis [7]. Specially, leptin is recently characterized as a linker between obesity and OA [8]. Mechanistically, leptin induces cartilage degradation by upregulating or activating matrix metalloproteinases [9,10] and proinflammatory cytokines, including IL-6, IL-8 and so on [11].

Vascular cell adhesion molecules-1 (VCAM-1), an inducible surface glycoprotein that belongs to immunoglobulin gene superfamily (IgSF) [12], was newly identified as a predictor of severe OA of the hip and knee joints [13]. It has been reported that VCAM-1 gene was transcriptionally regulated in a cell type-dependent manner [13]. Located near the transcriptional initiation site are several sequences that corresponded to previously defined transcription factor binding sites for NF-κB, GATA family [14], AP-1 [15] and Ets class [16]. Most recently, VCAM-1 was reported as a target of leptin [17], while the regulatory mechanism is still not fully characterized. In the present study, we identified a novel transcription factor CCAAT-enhancer-binding protein-α (C/EBP-α) responsible for leptin-induced VCAM-1 production by binding to the promoter region of VCAM-1, providing a possible molecular mechanism linking obesity to OA.

2. Materials and methods

2.1. Mice

All animal studies were conducted in accordance with the guidelines for the care and use of laboratory animals and were approved by the Animal Care and Use Committee. Male C57BL/6 mice were purchased from the experimental animal center of the Third Military Medical University. The mice were housed five per cage supplied with a normal rodent diet or high fat diet (HFD) in a pathogen-free facility with 12-h light, 12-h dark cycle. 12-week old

Abbreviations: C/EBP-α, CCAAT-enhancer-binding protein-α; VCAM-1, vascular cell adhesion molecules-1; OA, osteoarthritis

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mice with normal diet were subjected to primary chondrocyte isolation. The mice with 16-week HFD or normal diet were used for plasma leptin assay (#MOB00, R&D) and protein assay of primary chondrocytes.

2.2. Isolation of primary mouse and human chondrocytes

Primary mouse articular chondrocytes were isolated by dissection of the tibial plateaus and femoral condyles of the male C57BL/6 mice at 4 months of age. The articular cartilage was carefully peeled off with a scalpel under a dissecting microscope to avoid disruption of the subchondral bone. The cartilage was subsequently digested with 2 mg/ml clostridial collagenase (#17101-015, Invitrogen) at 37 °C for 2 h and then plated in monolayer culture. The chondrocytes from 5 mice were pooled as one sample upon isolation. The primary chondrocytes were directly subjected to protein assay or cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1% glutamine, 100 units/ml penicillin, 50 g/ml Streptomycin (Invitrogen), maintained and proliferated at 37 °C in the presence of 5% CO_2 for 7 days prior to the initiation of each experiment.

Normal human articular cartilage samples were obtained from the knee joints of patients undergoing total knee replacement surgery (with permission from the patients and the local ethics committee). The primary human chondrocytes were obtained following the protocol for primary mouse chondrocytes.

2.3. Protein extraction and immunoblotting

Cell proteins were extracted with RIPA Lysis Buffer and quantified by the BCA kit (Roche, USA). The extracted proteins were separated by 8% or 10% SDS–PAGE and transfered to a polyvinylidene difluoride membrane. The membrane was then used for immunoblotting with the antibodies [Anti-GAPDH (#2118, Cell signaling), Anti-C/EBP-α (sc-365318X, Santa Cruz), Anti-VCAM-1 (ab134047, Abcam)].

2.4. Realtime qPCR

Total RNA was extracted using the Trizol reagent (Invitrogen) according to the manufacture's protocol. Reverse transcription and quantitative PCR were performed as described previously [18]. All the primers used for PCR are available upon request.

2.5. ChIP assay

ChIP assay was used to study leptin-induced interaction between the C/EBP-α protein and VCAM-1 promoter DNA in mouse and human chondrocytes. The detailed protocol was described previously [19]. Briefly, the primers for C/EBP-α protein binding site in mouse VCAM-1 promoter were designed as: forward: 5′-AGACCTACAGAGGCATTT-3′, reverse: 5′-GAGATGAATTGACAACCA-3′. The primers for C/EBP-α protein binding site in human VCAM-1 promoter were designed as: forward: 5′-TGGGCAAGCATTTTGCAAGGCCTT-3′, reverse: 5′-CTTTTCCCCCCAGTGAAGTGCCTT-3′. Amplification conditions were denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s for 38 cycles, and the expected products were 100 bps.

2.6. Gain or loss of function studies

Mouse or human C/EBP-α overexpression was performed by transfecting the chondrocytes with a constitutive expression plasmid specific for mouse (MR205482, ORIGENE) or human C/EBP-α (RC218955, ORIGENE). Mouse or human endogenous C/EBP-α was knocked down by using a commercial siRNA kit (#SI00948311, QIAGEN) or (sc-37047, Santa Cruz), respectively.

2.7. Molecular cloning experiments

The experiments for DNA constructs, cell transfection, reporter gene assays and site-directed reporter gene mutation were performed as described previously [19].

2.8. Statistics

Data are generated as mean ± S.D. Comparisons were performed using ANOVA for multiple groups or Student's *t* test for two groups. $P < 0.05$ is considered statistically significant.

3. Results

3.1. Leptin induces mouse VCAM-1 production

To investigate the effect of leptin on VCAM-1 expression in chondrocytes, the primary mouse chondrocytes were treated with leptin in different concentrations and time points. As shown in Fig. 1, leptin stimulated VCAM-1 mRNA and protein levels in a time- and dose-dependent manner. The optimal condition of leptin for VCAM-1 stimulation is 500 nM for 24 h.

3.2. Leptin-induced VCAM-1 promoter activity via a novel C/EBP-α binding site

As leptin displayed stimulation on VCAM-1 production in mRNA and protein levels, we speculated that transcriptional mechanism would involve in this process. Therefore, a 3050-bp (−3000 to +50) DNA fragment harboring the mouse VCAM-1 promoter was cloned into pGL4-basic vector (PV1). Further, a series of mutated plasmids harboring different regions of VCAM-1 promoter (PV2–PV6) were constructed (Fig. 2A). Reporter gene assay indicated that promoter region −1000 to −500 bps is required for leptin-induced VCAM-1 transcriptional activity (Fig. 2B). Interestingly, the sequences −850 to −600 bps in mouse VCAM-1 gene are highly conserved, comparing to the sequences −950 to −700 bps in human VCAM-1 gene according to DNA sequence alignment (data not shown), which indicates that some important cis-acting elements locate in this region.

To identify the involved transcriptional factors in leptin-induced VCAM-1 transcription, we searched the putative transcription factor binding sites (TFBS) in DNA sequences from −850 to −600-bp in mouse VCAM-1 gene by using an online software (http://alggen.lsi.upc.es). Several elements including a p53 biding site (−683–689), a AP-2-α binding site (−637 to −642) and a C/EBP-α binding site (−617 to −623) got high scores. To identify whether those elements are functional in leptin-induced VCAM-1 expression, we performed site-directed mutations of those elements, respectively (Fig. 2C). Reporter gene assay experiments verified that the core sequences of C/EBP-α (but not p53 or AP-2-α) binding element (AATTGAG), is largely required for leptin-induced VCAM-1 promoter activity (Fig. 2D).

3.3. C/EBP-α involvement in leptin-induced VCAM-1 production

To confirm the regulatory role of C/EBP-α on VCAM-1 expression, we overexpressed or downregulated C/EBP-α in primary mouse chondrocytes, and determined the VCAM-1 expression. As expected, constitutive expression of C/EBP-α increased VCAM-1 expression in the levels of protein (Fig. 3A) and mRNA (Fig. 3D). Consistently, small interfering RNA (si-RNA)-mediated C/EBP-α

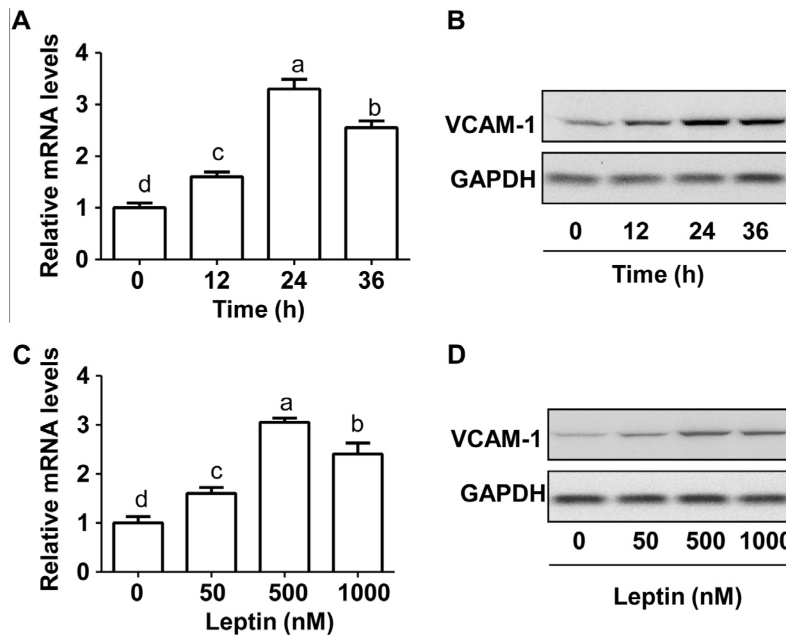

Fig. 1. Leptin induces VCAM-1 expression in primary mouse chondrocytes. Leptin stimulated mRNA (A) and protein (B) levels of VCAM-1 in a time-dependent manner as indicated. The primary mouse chondrocytes were treated with leptin (500 nM) for 0, 12, 24 and 36 h, and then subjected to mRNA and protein assay by realtime qPCR and Western blotting, respectively (A and B). Leptin induced mRNA (C) and protein (D) levels of VCAM-1 in a dose dependent manner. The primary mouse chondrocytes were treated with leptin for 24 h at different concentrations (0, 50, 100 and 500 nM). In *A* and *C*, values not sharing a common superscript letter differ significantly ($P < 0.05$, $n = 5$).

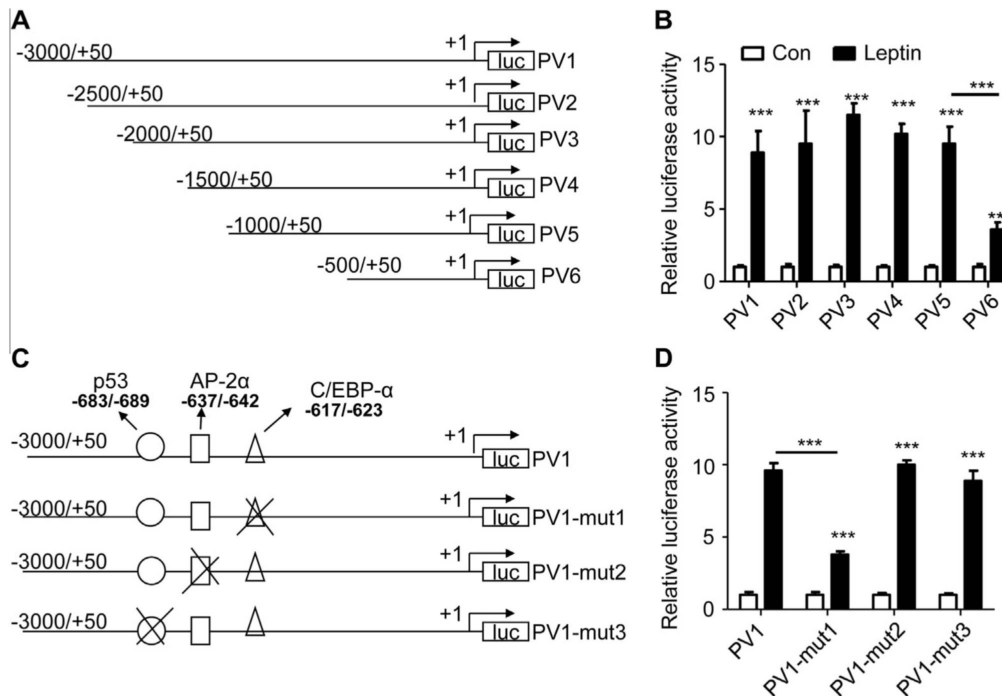

Fig. 2. Leptin induces VCAM-1 promoter activity via a C/EBP-α element in primary mouse chondrocytes. (A) A schematic depiction of different mouse VCAM-1 promoter regions cloned into pGL4-basic plasmid. The constructs were designed as PV1–PV6 as indicated. (B) Promoter region −1000 to −500-bp was required for leptin-induced luciferase activity of VCAM-1 in primary mouse chondrocytes according to the transfection and reporter gene assay ($^{***}P < 0.005$, $n = 5$). (C) The predicted C/EBP-α, AP-2α and p53 binding sites in the VCAM-1 promoter were mutated, respectively. A predicted C/EBP-α binding site (triangle) located at −617/−623, a AP-2α binding site (square) located at −637 to −642, and a p53 element (circle) located at −683 to −689 upstream of the transcription starting site of mouse VCAM-1 gene. The constructs PV1-mut1, PV1-mut2 and PV1-mut3 were designed from PV1 with the mutated elements of C/EBP-α, AP-2α and p53, respectively. (D) Mutation of the C/EBP-α binding site decreased leptin-induced promoter activity in primary mouse chondrocytes according to the transfection and reporter gene assay ($^{***}P < 0.005$, $n = 5$).

knockdown deceased VCAM-1 expression (Fig. 3B and E). Notably, C/EBP-α mRNA and protein levels were stimulated by leptin treatment. The stimulation of leptin on VCAM-1 production was largely rescued by C/EBP-α knockdown (Fig. 3C and F). Significantly, loss or gain of function of C/EBP-α in primary human chondrocytes deceased or increased VCAM-1 expression in protein levels, respectively (Fig. S1A and B). Small interfering RNA-mediated C/EBP-α knockdown largely abolished leptin-induced expression of

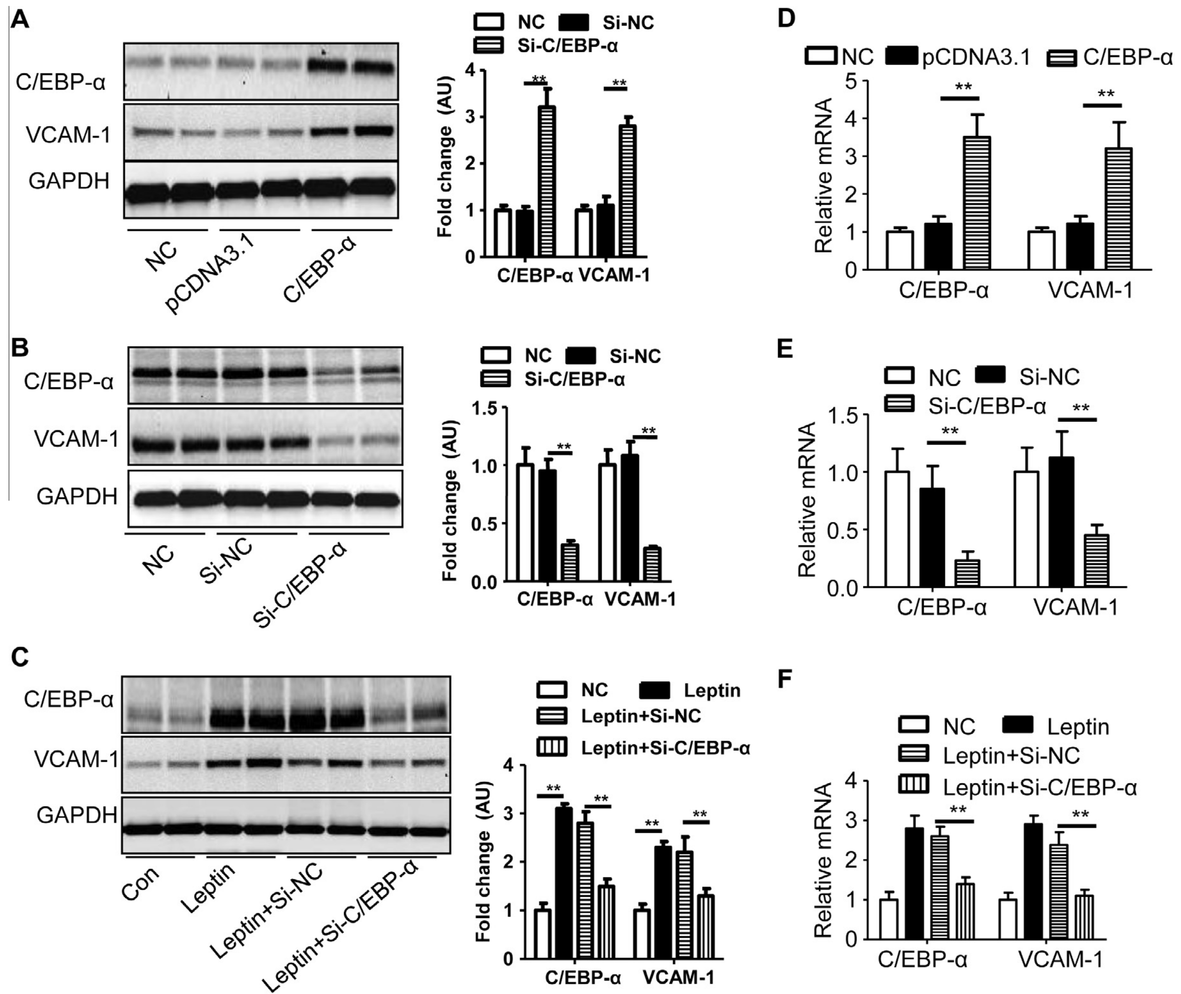

Fig. 3. C/EBP-α mediates leptin-induced VCAM-1 production in primary mouse chondrocytes. (A) Primary mouse chondrocytes were transfected with a plasmid expressing constitutive active C/EBP-α (0.4 μg/ml medium) or empty vector pCDNA3.1 as control for 24 h. The untreated cells were used as negative control (NC). Cell lysates were subjected to Western blotting assay for C/EBP-α, VCAM-1 and GAPDH as internal control (**$P < 0.01$, $n = 3$). (B) Primary mouse chondrocytes were transiently transfected with a small interfering RNA specific for mouse C/EBP-α (si-C/EBP-α) at a final dose of 20 nmol/ml or a scramble RNA as control (si-NC) for 36 h. The untreated cells were collected as negative control (NC). Cell lysates were analyzed as described in (A) (**$P < 0.01$, $n = 3$). (C) Primary mouse chondrocytes were transiently transfected with si-C/EBP-α (20 nmol/ml) or si-NC (20 nmol/ml) for 12 h, and then supplied with leptin (500 nM) for another 24 h. Cell lysates were analyzed as described in (A) (**$P < 0.01$, $n = 3$). (D) Primary mouse chondrocytes were treated as described in (A), and collected for the assay of C/EBP-α and VCAM-1 mRNAs by Realtime qPCR (**$P < 0.01$, $n = 5$). (E) Primary mouse chondrocytes were treated as described in (B), and collected for the assay of C/EBP-α and VCAM-1 mRNAs by Realtime qPCR (**$P < 0.01$, $n = 5$). (F) Primary mouse chondrocytes were treated as described in (C), and collected for the assay of C/EBP-α and VCAM-1 mRNAs by Realtime qPCR (**$P < 0.01$, $n = 5$).

C/EBP-α and VCAM-1 in human chondrocytes (Fig. S1C). Those data indicates that induction of leptin on C/EBP-α-dependent VCAM-1 expression is a common mechanism.

To further verify whether C/EBP-α protein directly bind to the predicted binding site in mouse VCAM-1 gene promoter, ChIP assay was performed. As shown in Fig. 4A, C/EBP-α could bind with the predicted elements located at −617 to −623. The binding activity could be potentiated by leptin treatment, and siRNA-mediated C/EBP-α knockdown attenuated this effect (Fig. 4B). Likewise, bioinformatical prediction indicated that a potential C/EBP-α binding site (AATTGGG) located at −792 to −786 in the conserved promoter region of human VCAM-1 gene. ChIP assay verified that leptin treatment could enhance C/EBP-α binding to the aforementioned binding site in human VCAM-1 gene (Fig. S1D).

3.4. Increased plasma leptin level correlated to increased VCAM-1 production in the mouse chondrocytes from diet-induced obese (DIO) mice

In order to correlate the regulatory mechanism in vitro to physiopathological significance in vivo, high-fat diet-induced obese

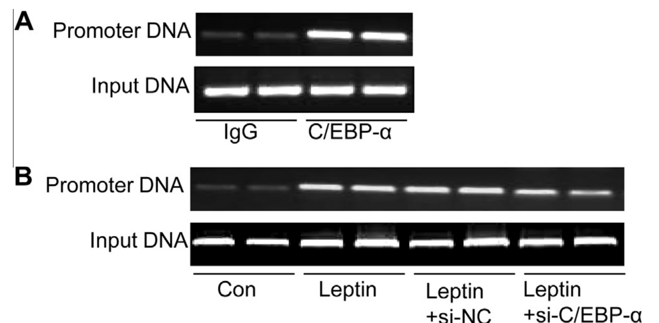

Fig. 4. Leptin induces C/EBP-α binding to VCAM-1 promoter. (A) Primary mouse chondrocytes were collected for ChIP assay. Cell lysates were immunoprecipitated with mouse anti-C/EBP-α antibody or IgG as control. (B) Primary mouse chondrocytes were transiently transfected with or without si-C/EBP-α (20 nmol/ml) or si-NC (20 nmol/ml) for 12 h, and then supplied with leptin (500 nM) for another 24 h. Cell lysates were subjected to ChIP assay as described in (A). Experiments in A and B were replicated for three times and representative results were displayed.

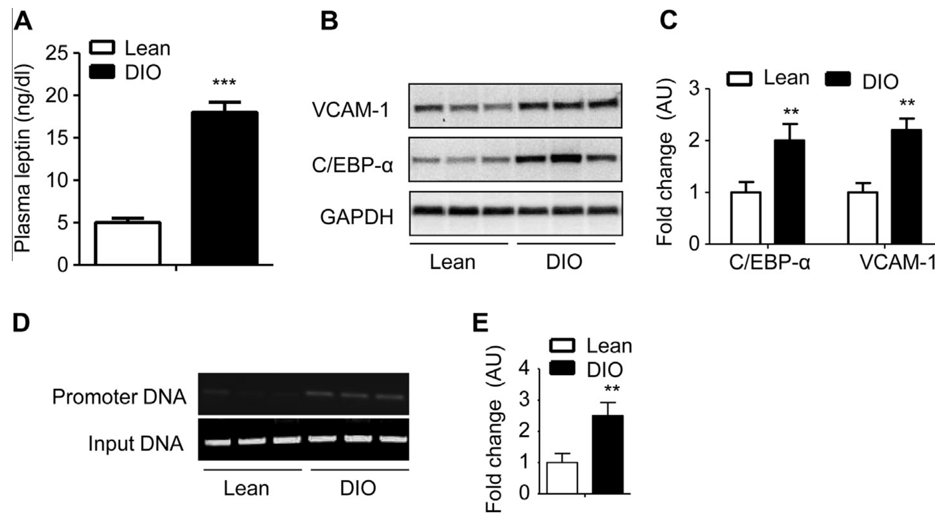

Fig. 5. Increased plasma leptin level is parallelized with increased C/EBP-α and VCAM-1 production in the mouse chondrocytes from diet-induced obese (DIO) mice. (A) Plasma leptin levels increased in the mice on a standard high fat diet for 16 weeks ($^{***}P < 0.005$, $n = 5$, compared with the age-matched lean mice). (B) DIO mouse-derived chondrocytes displayed increased C/EBP-α and VCAM-1 protein levels. (C) Statistic analysis of the results in B ($^{**}P < 0.01$, $n = 3$). (D) Increased binding of C/EBP-α to VCAM-1 promoter in DIO mouse-derived chondrocytes. (E) Statistic analysis of the results in D ($^{**}P < 0.01$, $n = 5$).

(DIO) mice model was employed. Plasma leptin level was notably increased (Fig. 4A) correlating to elevated C/EBP-α and VCAM-1 production in primary chondrocytes from DIO mice, as compared with the lean mice (Fig. 4B and C). To confirm the aforementioned mechanism, interaction between C/EBP-α protein and VCAM-1 promoter DNA was determined by ChIP assay. The relative DNA binding activity of C/EBP-α significantly increased by 1.5-fold in DIO mice-derived chondrocytes comparing to the ones form the lean mice (Fig. 5D and E).

4. Discussion

Obesity-related Osteoarthritis is a most common rheumatic disease, with increased proinflammatory cytokine production and immune cell infiltration in the joints. Thus, extravasation of leukocytes from circulating blood to inflamed tissue is crucial in inflammatory process and this complex event is regulated by VCAM-1 [20]. Blockade of VCAM-1 notably inhibited the binding and recruitment of leukocytes into inflamed joints [21,22]. VCAM-1 has been identified as a predictor of severe OA of the hip and knee joints [13]. Thus, it is very important to explore the regulatory factors of VCAM-1.

Chondrocytes are important resource of the cytokines or chemokines [23] and play a very crucial role in maintaining the cartilage structure. VCAM-1 is one of the cytokines expressed by chondrocytes [24], and could be modulated by leptin-induced JAK2, PI3K and AMPK signaling [17]. Here we described a novel transcriptional mechanism for leptin-mediated VCAM-1 production.

In previously studies, VCAM-1 was reported to be regulated by some transcriptional factors via the cis-acting elements which are located in the proximal region upstream of transcriptional binding site of VCAM-1 gene [14–16,25]. Here we identified a novel C/EBP-α binding site (AATTGAG) locating at −617 to −623 in the mouse VCAM-1 gene promoter. However, mutation of this element could not fully rescue leptin's induction on VCAM-1 promoter activity (Fig. 2A and B), indicating that some other well-identified cis-acting elements like NF-κB and AP-1 in the proximal region might also contribute to leptin-induced VCAM-1 promoter activity, because those transcriptional factors could be activated by leptin in other cell types [26,27]. Therefore, further studies are required to elucidate this hypothesis.

To explore the physiopathological significance, we finally confirmed the molecular mechanism in diet-induced obesity mice and found that elevated plasma leptin level was correlated to increased C/EBP-α, VCAM-1 production and interaction between C/EBP-α protein and VCAM-1 promoter DNA in primary chondrocytes. We propose that obesity-related leptin or other cytokines increase the transcriptional activity of C/EBP-α or other transcription factors like NF-κB to promote VCAM-1 production in chondrocytes. The over-produced VCAM-1 would promote the progression of obesity-related OA (Fig. 6).

Taken together, leptin induces VCAM-1 production by facilitating C/EBP-α binding to its corresponding binding site in VCAM-1 gene in mouse chondrocytes. Our findings provide a possible molecular mechanism linking obesity to OA and shed light on the new strategy for therapeutic intervention of obesity-related OA.

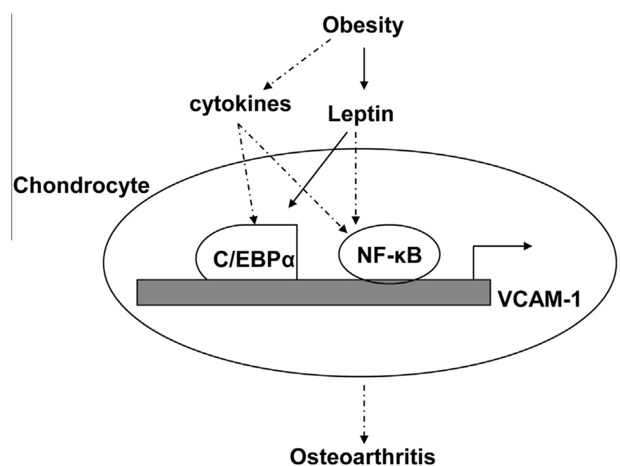

Fig. 6. Proposed molecular mechanism for leptin-mediated obesity-related osteoarthritis. Plasma leptin level increases in obesity, which activates C/EBP-α and/or other possible transcription factors like NF-κB binding to VCAM-1 promoter to promote VCAM-1 production in chondrocytes. Finally, increased VCAM-1 production aggravates obesity-related osteoarthritis (The solid arrow-indicated flow is verified in this work and the dotted arrow-indicated flow comes from previous studies.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.02.032.

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