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Leptin increases VEGF expression and enhances angiogenesis in human chondrosarcoma cells

Wei-Hung Yang [a,b,c,d], Jui-Chieh Chen [e,f], Kai-Hsiang Hsu [f], Chih-Yang Lin [f], Shih-Wei Wang [g], Shoou-Jyi Wang [h], Yung-Sen Chang [a], Chih-Hsin Tang [f,i,j,*]

[a] Department of Orthopedic Surgery, Taichung Hospital, Ministry of Health and Welfare, Taichung, Taiwan
[b] School of Chinese Medicine, China Medical University, Taichung, Taiwan
[c] Department of Nursing, National Taichung University of Science and Technology, Taichung, Taiwan
[d] Graduate Institute of Biotechnology, National Chung Hsing University, Taichung, Taiwan
[e] Department of Biochemical Science and Technology, National Chiayi University, Chiayi, Taiwan
[f] Graduate Institute of Basic Medical Science, China Medical University, Taichung, Taiwan
[g] Department of Medicine, Mackay Medical College, New Taipei City, Taiwan
[h] Department of Orthopedic Surgery, Chang-Hua Hospital, Ministry of Health and Welfare, Puhsin Township, Changhua County, Taiwan
[i] Department of Pharmacology, School of Medicine, China Medical University, Taichung, Taiwan
[j] Department of Biotechnology, College of Health Science, Asia University, Taichung, Taiwan

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ABSTRACT

Background: Leptin, 16 kDa product of obese gene, is adipocytokine playing critical role in regulation of body weight. In recent years, leptin is also defined as potent angiogenic factor involving in tumorigenesis, angiogenesis, and metastasis. However, it is unknown whether leptin regulates VEGF production in human chondrosarcoma and contributing the tumor-associated angiogenesis.
Methods: We analyzed protein level of leptin and VEGF in human chondrosarcoma tissues. Effects of leptin on chondrosarcoma cells were examined by in vitro and in vivo assays. In addition, intracellular signal pathways were investigated by pharmacological and genetic approaches.
Results: We found that both leptin and VEGF are highly expressed in human chondrosarcoma tissues, and positively correlated with tumor stage. Leptin increases VEGF production by activating OBRl receptor and MAPKs (p38, ERK, and JNK), which in turn enhances binding of AP-1 transcription factor to VEGF promoter, resulting in the transactivation of VEGF expression and subsequently promoting migration and tube formation in endothelial progenitor cells (EPCs). In vivo, knockdown leptin significantly reduces angiogenesis and tumor growth.
Conclusion: Leptin may be a therapeutic target of angiogenesis and metastasis in chondrosarcoma.
General significance: These findings provide better understanding of pathogenesis of chondrosarcoma and can utilize this knowledge to design new therapeutic strategy.

1. Introduction

Chondrosarcomas, second most frequent bone malignancy, are a heterogeneous group of neoplasms characterized by production of cartilage matrix. High-grade ones often metastasize, resulting in poor prognosis and lethality. To date, surgical resection remains the primary mode of treatment for chondrosarcomas, since conventional chemotherapy and radiotherapy are largely ineffective [1,2]. Previous studies have indicated that the grade of chondrosarcoma correlates with microvessel density and vascular endothelial growth factor (VEGF) expression [3–5].

VEGF (also referred to as VEGF-A) is well documented to have the ability to promote growth and survival of endothelial cells as well as induce their migration and organization into capillary-like tubes [6,7]. VEGF is the most important mediator of tumor angiogenesis, inducing formation of new blood vessels and providing routes by which tumors cells enhancing metastasis to distant sites [8]. VEGF-targeted strategies significantly attenuated angiogenesis and tumor growth [9–12]. VEGF is therefore an attractive target for cancer therapy to prevent disease progression.

Leptin, 16 kDa product of *ob* gene from adipocytes, is hormone regulating appetite, energy expenditure, and body weight. It exerts its physiological action via leptin receptor (OBR): e.g., long (OBRl) and short (OBRs) isoforms [13]. In recent years, leptin is also defined as potent angiogenic factor involved in tumorigenesis, angiogenesis, and metastasis [14–17]. Stimulation of endothelial cells with leptin led to increase in cell proliferation and/or survival and elicited marked

* Corresponding author at: Graduate Institute of Basic Medical Science, China Medical University, No. 91 Hsueh-Shih Road, Taichung, Taiwan. Tel.: +886 4 2205 2121 7726; fax: +886 4 22333641.
E-mail address: chtang@mail.cmu.edu.tw (C.-H. Tang).

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enhancement of angiogenesis [18,19]. Leptin also induced VEGF expression in some types of cancer, which may synergistically induce angiogenesis to accelerate invasion and metastasis of tumor cells [20–23]. In clinical specimens, level of leptin expression in different cancers is closely associated with degree of malignancy, including tumor size, metastasis, and poorer prognosis [24–26].

While roles of leptin have emerged as angiogenic factor or modulator for VEGF across diverse cancers, leptin expression in chondrosarcoma has not been investigated, with its exact role in development and promotion of cancer still largely unknown. We probed intracellular signal pathways involved in leptin-induced VEGF production in human chondrosarcoma and effects of leptin on inducing neovascularization, using in vitro and in vivo assays.

2. Materials and methods

2.1. Materials

Protein A/G beads, anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, antibodies specific to p-p38, p38, p-ERK, ERK, p-JNK, JNK, p-c-Jun, c-Jun, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); ON-TARGET plus siRNAs targeting p38, ERK, JNK, c-Jun, and control from Dharmacon Research (Lafayette, CO); VEGF antibodies from Abcam (Cambridge, MA); recombinant human VEGF from R&D Systems (Minneapolis, MN); U0126, SB203580, SP600125, curcumin, tanshinone, and PDTC from Calbiochem (San Diego, CA); all other chemicals from Sigma-Aldrich (St. Louis, MO).

2.2. Cell culture

Human chondrosarcoma cell line (JJ012) was kindly provided by the laboratory of Dr. Sean P. Scully (University of Miami School of Medicine, Miami, FL) and human chondrosarcoma cell line (SW1353) was purchased from the American Type Culture Collection, then cultured in Dulbecco's modified Eagle's medium (DMEM)/α-MEM supplemented with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin at 37 °C in humidified chamber in 5% CO_2.

2.3. Oligonucleotide transfection

JJ012 cells were transfected with phosphorothioate double-stranded decoy oligonucleotide (ODN) carrying either OBRl antisense ODN (AS-ODN; AGACCG AGCGGGCGTTAA) or missense ODN (MM-ODN; AGCC CGCGCGAGTGTTCA). ODN (5 μM) was mixed with Lipofectamine 2000 (10 μg/ml) for 30 min, then added to cells in serum-free medium. After 24 h transfection, cells were used for the following experiments.

2.4. Isolation and cultivation of circulating EPCs

Study protocol was approved by the Institutional Review Board of MacKay Medical College (New Taipei City, Taiwan) (reference number: P1000002); all subjects gave informed written consent before enrollment. After collecting peripheral blood from donors, mononuclear cells were isolated by density-gradient centrifugation with Ficoll-Paque plus (Amersham Biosciences, Uppala, Sweden). For $CD34^+$ selection, mononuclear cells were subjected to immunomagnetic separation using a CD34 MicroBead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) as per manufacturer's instructions. Human $CD34^+$ EPCs were maintained and propagated in MV2 complete medium, consisting of MV2 basal medium and growth supplement (PromoCell, Heidelberg, Germany), and supplied with 20% defined fetal bovine serum (HyClone, Logan, UT). Cultures were seeded onto 1% gelatin-coated plasticware and maintained at 37 °C in a humidified atmosphere of 5% CO_2.

2.5. Immunohistochemistry (IHC)

Human chondrosarcoma tissue array was purchased from Biomax (Rockville, MD; 6 cases for normal cartilage, 24 cases for Grade I, 9 cases for Grade II, and 15 cases for Grade III chondrosarcoma). Tissues were mounted on glass slides, rehydrated, and incubated in 3% H_2O_2 to block endogenous peroxidase activity. Sections were incubated in 3% bovine serum albumin in phosphate-buffered saline (PBS) to block nonspecific reaction, and then primary mouse monoclonal anti-human leptin or VEGF antibody applied to slides at 1:50 dilution. After incubation overnight at 4 °C in a humidified chamber, slides were washed and incubated with appropriate secondary antibodies and staining was revealed by ImmPACT DAB kit (Vector Laboratories, Burlingame, CA). Slides were counterstained in hematoxylin, dehydrated, and mounted. Sum of intensity and percentage was used as final staining scores (0–5).

2.6. Quantitative real-time PCR

Total RNA extracted by TRIzol reagent (MDBio Inc., Taipei, Taiwan) was reverse transcribed into cDNA using M-MLV reverse transcriptase, Oligo (dT), and dNTP Mix (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Synthesized cDNA (100 ng) served as template for quantitative real-time PCR, conducted with a TaqMan One-step PCR Master Mix Kit. Sequences for target gene primers and probes were obtained commercially and qPCR assayed in triplicate by StepOnePlus Sequence Detection System (all from Applied Biosystems, Foster City, CA). Relative gene expression calculated from glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression value.

2.7. Western blot analysis

Cells were lysed in RIPA buffer containing protease inhibitor cocktail and protein concentration was determined by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Inc., Waltham, MA). Equal amount of total protein was resolved by SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA). Membranes blocked with 4% bovine serum albumin were probed with primary antibodies. Washed membranes incubated with peroxidase-coupled secondary antibodies were washed again, bound antibodies visualized by ECL reagents (PerkinElmer, MA) and autoradiography.

2.8. Chromatin immunoprecipitation (ChIP) assay

ChIP analysis was performed as described previously [27]. Briefly, cells were fixed with 1% formaldehyde, washed, and lysed. Cell lysates were sonicated to shear DNA into smaller fragments. Protein–DNA complexes were precipitated with anti-c-Jun antibody. After reverse cross-link of protein–DNA complexes, free DNA was then extracted with phenol-chloroform. Purified pellet was subjected to PCR, products resolved by 1.5% agarose gel electrophoresis and visualized by UV light. Primers 5′-CCTTTGGGTTTTGCCAGA-3′ and 5′-CCAAGTTTGTGGAGCT GA-3′ were utilized to amplify across the VEGF promoter region.

2.9. Luciferase reporter assay

JJ012 cells were co-transfected with luciferase reporter gene constructs and β-galactosidase by Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. After 24 h transfection, cells were exposed to various doses (10–300 nM) of leptin for 24 h or pre-treated with inhibitors for 30 min, followed by treatment with leptin for 24 h, or transfected with siRNAs, followed by treatment with leptin for 24 h. Luciferase activity was determined using the luciferase assay kit (Promega, Madison, MA).

2.10. Preparation of conditioned media

JJ012 cells (2×10^6) were grown overnight in 10-cm tissue dishes in cell culture medium. After two PBS washes, cells were incubated in serum-free medium for 48 h before collection of conditioned medium (CM). To normalize for density differences due to proliferation during culture period, cells from each plate were collected and total DNA content/plate determined (spectrophotometric absorbance, 260 nm). CM was then normalized by DNA content among samples.

2.11. Tube formation assay

Surface of 48-well plates was coated with 150 μl of Matrigel matrix (BD Biosciences, Bedford, MA), allowed to polymerize at 37 °C for 30 min. EPCs (5×10^4 cells/well) were cultured in 100 μl cultured media (50% EGM-MV2 media and 50% CM). After 16 h incubation at 37 °C, photomicroscope assessed EPC tube formation, each well photographed and five random fields counted at ×200 magnification. Tube branches and lengths were measured by MacBiophotonics Image J software (Bethesda, MD).

2.12. Chick chorioallantoic membrane (CAM) assay

To gauge effect of leptin on tumor-induced angiogenesis, CAM assay was carried out as described earlier [28]. Briefly, fertilized chicken eggs were incubated at 38 °C in 80% humidified atmosphere. On Day 8, CM from JJ012/control-shRNA or JJ012/leptin-shRNA (2×10^4 cells) was applied to chorioallantoic membrane. After 48 h incubation, membranes underwent microscopy and photographic documentation. Angiogenesis was quantified by counting blood vessel branches, at least 10 viable embryos tested for each treatment. VEGF and PBS served as positive and negative controls, respectively. Animal protocols were approved by China Medical University (Taichung, Taiwan) institutional animal care and use committees.

2.13. Xenograft mouse model

Four-week-old male nude mice were randomly assigned to two groups. Cells (JJ012/control-shRNA or JJ012/leptin-shRNA) were resuspended in 100 μl of serum-free DMEM/α-MEM and subcutaneously injected (1×10^6 cell per mouse) into right flanks. Tumor growth was monitored using in vivo imaging system (Xenogen IVIS imaging system). At Week 7, mice were sacrificed by overdose with anesthetic. Tumors were removed, photographed, and weighed; tumor volume was calculated as ($1/2$ [length × width^2]). Tumors were partly measured the amount of hemoglobin using the Drakin's reagent kit (Sigma-Aldrich, St. Louis, MO) to quantify formation of blood vessels. The other fixed in 10% formalin, embedded in paraffin and subsequently processed for IHC staining with CD31.

2.14. Matrigel plug assay

Angiogenesis was assayed as detailed earlier [29]: nude mice subcutaneously injected with 400 μl of Matrigel alone or mixed with CM from either JJ012/control-shRNA or JJ012/leptin-shRNA. At Day 7, plugs were excised, photographed, and processed for IHC; paraffin sections immunostained with CD31.

2.15. Statistics

All data are presented as mean ± SEM. Statistical comparison of two groups used Student's *t*-test; for more than two groups, we used one-way analysis of variance with Bonferroni's post hoc test. In all cases, $P < 0.05$ was considered significant.

3. Results

3.1. Leptin and VEGF expressions positively correlate with degree of malignancy

To rate clinical significance of leptin and VEGF in chondrosarcoma cases, we utilized tissue array for evaluation by IHC to compare leptin and VEGF expression in normal cartilage and different chondrosarcoma grades. Fig. 1A shows representative samples of IHC staining for leptin and VEGF in normal cartilage and chondrosarcoma tissues with diverse grades. Expression of leptin and VEGF markedly increased with tumor stage (Fig. 1B–C). Quantitative data shows leptin expression positively correlated with VEGF expression levels in human chondrosarcoma patients (Fig. 1D).

3.2. Leptin/OBRl axis induces VEGF expression to drive activity of angiogenesis

To clarify direct relationship between leptin and VEGF in angiogenic process, we next examined whether leptin regulates VEGF expression in human chondrosarcoma. Fig. 2A shows leptin augmented VEGF mRNA expression in a dose-dependent manner; it manifestly raised VEGF protein levels, as assessed by Western blot and ELISA (Fig. 2B). To evaluate CM effect from leptin-treated chondrosarcoma cells on angiogenesis, in vitro angiogenesis model used cell migration and tube formation of EPCs. Stimulation of EPCs with CM increased migration and tube formation in EPCs dose-dependently (Fig. 2C–D). To confirm VEGF as most active influential factor in CM, we tested for neutralization of VEGF with specific antibody reducing migration and tube formation in EPCs. Results revealed that effects of CM on angiogenesis were diminished in the presence of neutralizing antibodies (Fig. 2C–D). We next examined leptin receptor involvement in leptin-mediated increase of VEGF production in chondrosarcoma cells. OBRl receptor mRNA expression increased in chondrosarcoma cells after treatment with leptin for 24 h, yet leptin did not affect mRNA levels of OBRs receptor (Fig. 2E). Further experiments revealed chondrosarcoma cells transfected with OBRl AS-ODN but not MM-ODN attenuating leptin-induced VEGF yield (Fig. 2F). These indicate leptin/OBRl-dependent signal enhancing EPCs angiogenesis in vitro by up-regulating VEGF in human chondrosarcoma cells.

3.3. MAPK pathways involved in leptin-mediated VEGF expression and angiogenesis

Mitogen-activated protein kinases (MAPKs) demonstrably mediate a gamut of cell behaviors, being an attractive target for therapeutic applications in cancer [30]. We first investigated whether leptin activates ERK, p38, and JNK. Fig. 3A shows treatment of JJ012 cells with leptin spawned time-dependent phosphorylation of ERK, p38, and JNK. To ascertain MAPK pathways involved in leptin-mediated VEGF expression and angiogenesis, JJ012 cells were pre-treated with the ERK, p38, or JNK inhibitors before leptin was added. Leptin-induced VEGF expression, at both mRNA and protein levels, was appreciably down-regulated in the presence of inhibitors in JJ012 cells, as assessed by qPCR (Fig. 3B), Western blot (Fig. 3C, top), and ELISA (Fig. 3C, bottom). Likewise, effects of CM from leptin-treated chondrosarcoma cells on EPCs migration and tube formation was attenuated by ERK, p38, or JNK inhibitor (Fig. 3D–E). To validate contribution of MAPK pathways to leptin-induced VEGF expression and angiogenesis, knockdown of MAPK genes expression in JJ012 cells was performed with ERK, p38, or JNK siRNA. Indeed, transfection with ERK, p38, or JNK siRNA significantly decreased leptin-induced VEGF expression at both mRNA and protein levels compared with normal control, as assessed by q-PCR (Fig. 3F) and ELISA (Fig. 3G, bottom). Knockdown efficiency of MAPK genes was verified by Western blot (Fig. 3G, top). CM from siRNA-transfected JJ012 cells assessed EPC migration and tube formation; data affirmed that knockdown of MAPK genes reduced CM-mediated cell migration and tube formation (Fig. 3H, I),

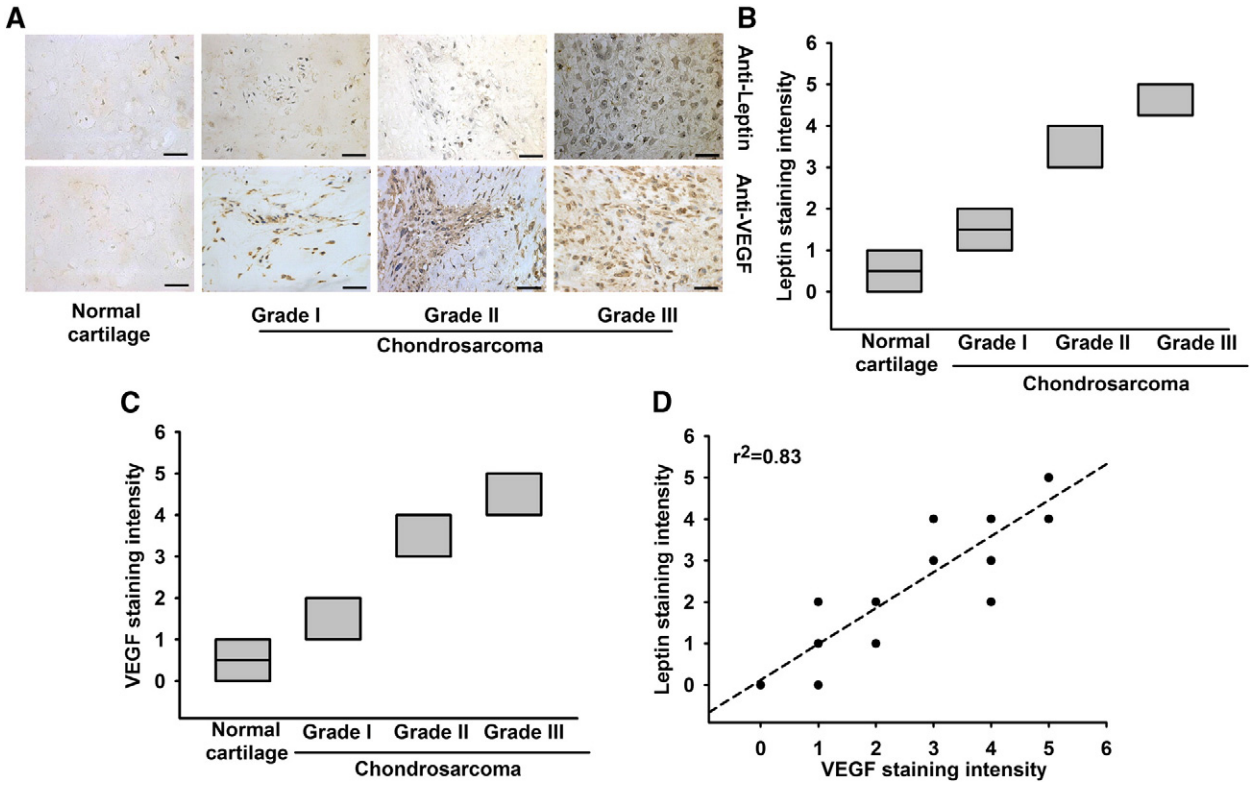

Fig. 1. Leptin and VEGF expression positively correlate with histopathological grade in human chondrosarcoma. (A) IHC for leptin and VEGF in representative samples of normal cartilage and different grades of chondrosarcoma tissue (grades I–III). Box plot comparing expression levels of leptin (B) and VEGF (C) in each histological grade of chondrosarcoma. The maximum, average, and minimum score represent the upper, middle, and lower line in the boxes, respectively. (D) Scatter plot shows correlation between leptin and VEGF for tissues noted in diverse grades of chondrosarcoma. R = 0.83.

suggesting MAPK activation involved in leptin-induced VEGF expression and angiogenesis.

3.4. Transcription factor AP-1 requires for leptin-mediated VEGF expression in human chondrosarcoma cells, subsequently eliciting angiogenic activities

Prior studies have indicated that c-Jun, component of AP-1 (activating protein-1), can be phosphorylated by MAPKs, leading to significant increase in the activity of AP-1 [31]. Additionally, leptin reportedly activates AP-1 [32]. We thus hypothesized AP-1 as involved in leptin-mediated VEGF expression in human chondrosarcoma cells. Our data demonstrated that leptin induced significant increase in c-Jun phosphorylation (Fig. 4A), but this effect was attenuated by U0126, SB203580, and SP600125 (Fig. 4B). Similarly, leptin-induced increase of VEGF expression, at both mRNA and protein levels, was drastically inhibited either in the presence of AP-1 inhibitors (curcumin and tanshinone) or in cells transiently transfected with c-Jun siRNA (Fig. 4C–D). Knockdown efficiency of c-Jun was verified by Western blot (Fig. 4C, top). Furthermore, we have also found that CM of leptin-stimulated JJ012 cultures elicited significant rises in EPC migration and tube formation (Fig. 4E–F). We next explored whether leptin activates MAPK pathway leading to transcriptional activation of VEGF through binding to the functional AP-1 site. The in vivo recruitment of c-Jun to the VEGF promoter was assessed by ChIP assay. As shown in Fig. 4G, in vivo binding of c-Jun to AP-1 element of VEGF promoter occurred after leptin stimulation, but this binding was attenuated by U0126, SB203580, and SP600125. To further confirm that AP-1 element is involved in leptin-induced VEGF expression, we performed promoter activity assays using transient transfection with AP-1 responsive luciferase construct into JJ012 cells. After treatment of JJ012 cells with leptin showed a dose-dependent increase in AP-1 promoter activity (Fig. 4H). Increase in AP-1 activity induced by leptin was antagonized by U0126, SB203580, and SP600125 as well as by ERK, p38, and JNK siRNA (Fig. 4I–J). These findings suggest that AP-1 is required for leptin-induced up-regulation of VEGF expression in human chondrosarcoma cells.

3.5. Leptin knockdown reduced VEGF expression and angiogenesis in vitro and in vivo

In addition to exogenous leptin treatment, we used a loss-of-function approach to assess effects of leptin knockdown on VEGF expression. We established two stable cell lines in JJ012 by infecting control shRNA or leptin shRNA, followed by selection with puromycin. As shown in Fig. 5A, leptin protein expression was significantly reduced by leptin shRNA in JJ012 cells, as compared with control shRNA. Consistent with leptin treatment, our data show VEGF expression at mRNA and protein level dramatically downregulated in JJ012 cells harboring leptin shRNAs versus those carrying control shRNA (Fig. 5A, B). Moreover, we measured VEGF protein expression in culture medium of these stable cell lines and found that VEGF protein concentration was significantly lower in JJ012 cells expressing leptin shRNA versus control shRNA (Fig. 5C). To further confirm effects of leptin on angiogenesis, we analyzed its angiogenic activities in vitro using CM from JJ012 cells with leptin knocked down. Results revealed that knockdown of leptin reduced CM-mediated tube formation and cell migration in EPCs. (Fig. 5D, E). In addition, we evaluated effect of leptin on angiogenesis using the in vivo model of CAM assay. CM from JJ012/leptin-shRNA decreased angiogenesis in CAM compared with that of CM from JJ012/control-shRNA (Fig. 5F). Ability of leptin to promote in vivo induced angiogenesis was next examined using mouse Matrigel plug assay. Mice were subcutaneously injected with Matrigel alone or mixed with CM from JJ012/control-shRNA and JJ012/leptin-shRNA. At Day 7, formed Matrigel plugs in mice were excised and photographed. Plugs with CM from JJ012/control-shRNA displayed greater neovascularization. By contrast, plugs with Matrigel alone and mixed CM from JJ012/leptin-shRNA have little or no blood vessel

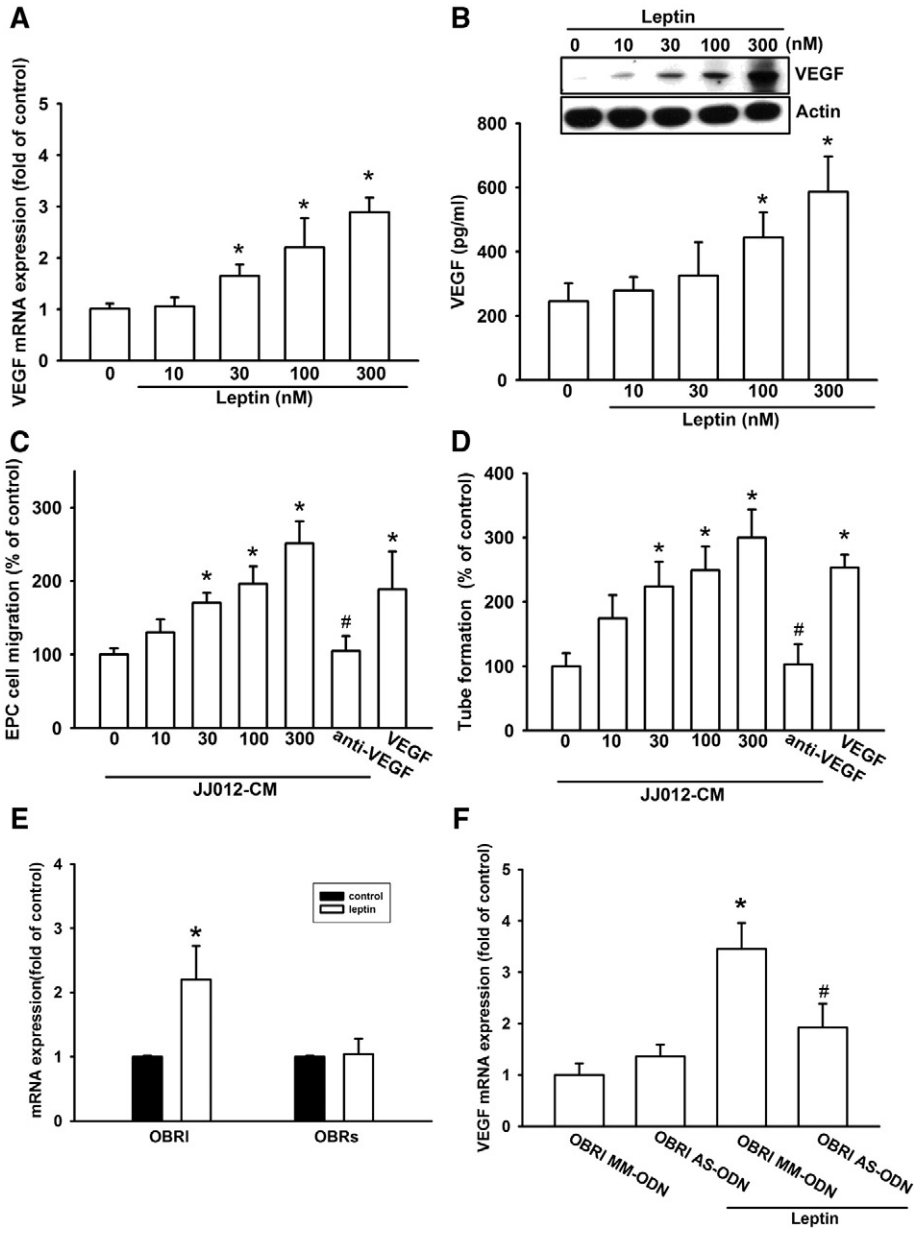

Fig. 2. Leptin promotes VEGF expression in chondrosarcoma cells via OBRl receptor and subsequently stimulates EPC migration and tube formation. (A) VEGF mRNA expression was assessed by qPCR in JJ012 cells after incubation with designated leptin concentrations for 24 h (n = 5). (B) VEGF protein expression was examined by Western blot (top) and ELISA (bottom) (n = 4). (C–D) JJ012 cells were pre-treated with/without anti-VEGF or VEGF for 30 min prior to stimulation with leptin (300 nM) or incubated with leptin (10–300 nM) for 24 h. The CM were collected and applied to EPCs for 24 h, CM effect on cell migration and capillary-like structure formation in EPCs examined by Transwell (n = 3) and tube formation assay (n = 6). (E) JJ012 cells were incubated with leptin (300 nM) for 24 h, OBRl and OBR mRNA expression examined by qPCR (n = 6). (F) JJ012 cells transfected with OBRl MM-ODN or OBRl AS-ODN for 24 h were treated with leptin (300 nM) for 24 h. RNA was isolated and relative VEGF mRNA levels detected by qPCR (n = 6), results expressed as means ± SEM. $^{*}P < 0.05$ compared to control; $^{\#}P < 0.05$ compared to leptin-treated group.

formation (Fig. 5G upper panel). Histologic section of plugs shows CD31-positive vessels starkly decreasing by knockdown of leptin (Fig. 5G, lower panel). These augur leptin promoting VEGF expression and neovessel formation in vitro and in vivo.

3.6. Knockdown of leptin attenuates tumor angiogenesis and arrests chondrosarcoma tumor growth in vivo

To assess effect of leptin on in vivo tumorigenesis, we used xenograft mouse model with human chondrosarcoma cells transduced with lentiviral shRNA against leptin or control. The cancer cells were labeled with firefly luciferase to monitor tumor growth. Tumorigenicity was assessed weekly by luciferase bioluminescence. Fig. 6A shows JJ012/leptin-shRNA-bearing mice with strongly suppressed tumor growth compared with JJ012/leptin-control across time. Tumors were excised and photographed at Day 42 after tumor cell implantation (Fig. 6B); those from JJ012/leptin-shRNA-bearing mice had much smaller size and weight than those from JJ012/leptin-control mice (Fig. 6C–D). Tissue hemoglobin content has been used as an alternative marker of angiogenesis [33,34]. The hemoglobin in tumors was much lower in JJ012/leptin-shRNA mice than that in JJ012/leptin-controls (Fig. 6E). Knockdown of leptin also decreased number of blood vessels in tumors, as assessed by staining of $CD31^{+}$ microvessels (Fig. 6F). Analyses of linkage between hemoglobin content and tumor volume revealed strong

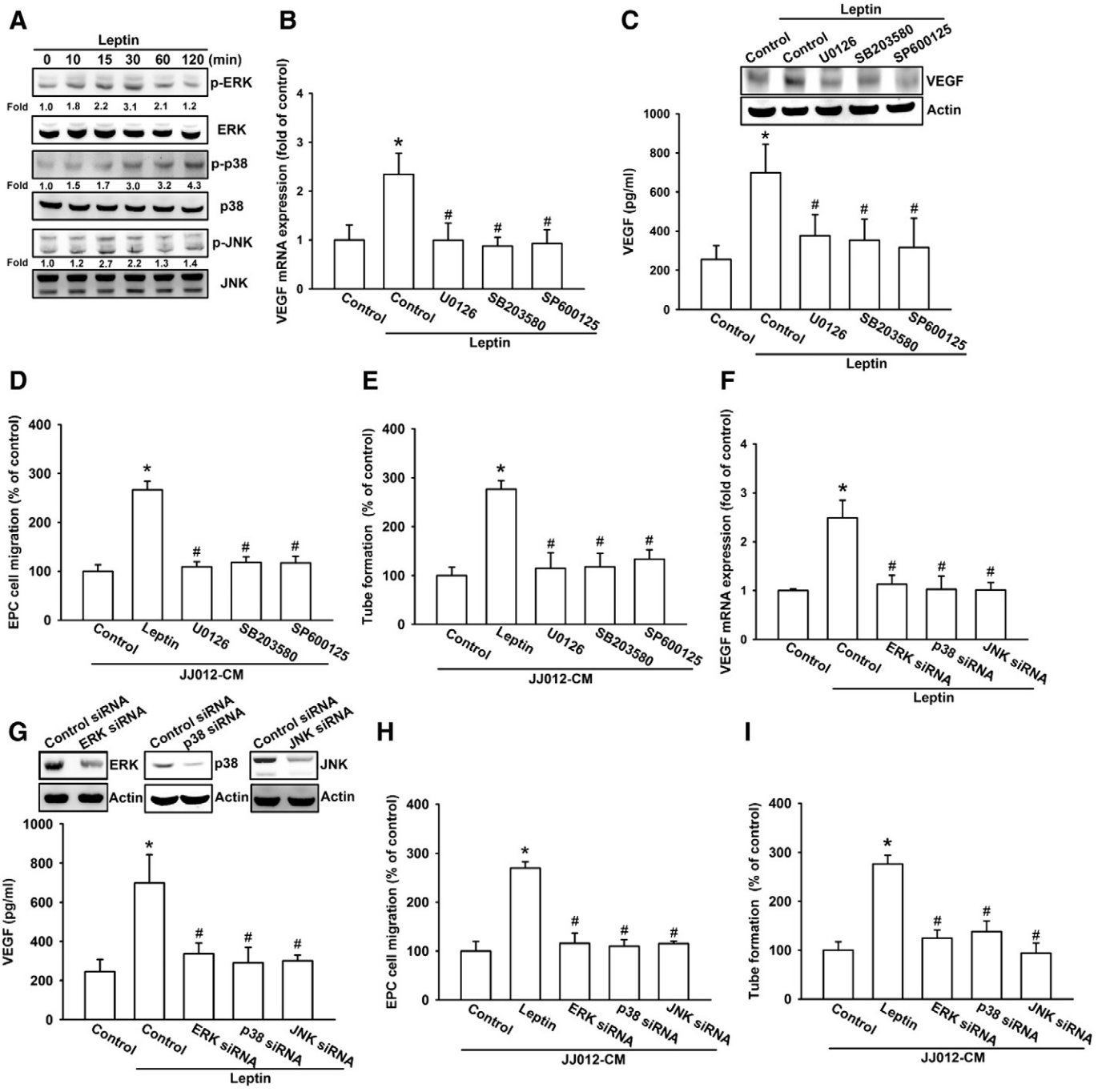

Fig. 3. MAPKs (ERK, p38, and JNK) are involved in leptin-induced VEGF production and angiogenesis in human chondrosarcoma. (A) JJ012 cells were incubated with leptin for indicated time intervals and phosphorylation of ERK, p38, and JNK ascertained by Western blot. JJ012 cells were pre-treated with the U0126 (10 μM), SB203580 (10 μM), or SP600125 (10 μM) for 30 min, followed by treatment with leptin for 24 h. VEGF mRNAs were monitored by q-PCR (n = 5) (B), and VEGF protein levels were assessed by Western blot (top) and ELISA (bottom) (n = 4) (C). (D–E) The same procedure was carried out with JJ012 cells; CM was collected and applied to EPCs for 24 h. Effect of CM on cell migration and capillary-like structure formation in EPCs was examined by Transwell (n = 3) and tube formation assay (n = 6). (F) JJ012 cells transfected with ERK, p38, or JNK siRNA for 24 h were stimulated with leptin for 24 h. VEGF mRNAs were examined by qPCR (n = 5). (G) VEGF protein expression was rated by ELISA (bottom) (n = 4); Western blot verified siRNA knockdown efficiency (top). (H-I) CM from siRNA-transfected JJ012 cells were used to assess EPC migration (n = 3) and tube formation (n = 6).

positive correlation (Fig. 6G). Overall, these aver leptin's role in VEGF-induced angiogenesis and tumor growth in vivo.

4. Discussion

Recent years have seen obesity linked with high risk of malignancy and elevated death rates from various cancers [35,36]. High-level expression of leptin is observed in obese humans as well as in genetic and environmentally induced forms of rodent obesity [37]. These relationships indicate leptin as pivotal in pathogenesis of human neoplasms. In best our knowledge, current study is a first time to examine the expression of leptin in human chondrosarcoma. We found the expression of leptin was correlated with tumor stage and was significantly higher than that in the normal cartilage. Therefore, leptin may be a biomarker for disease progression of chondrosarcoma. However, the relationship between obesity/serum leptin level and the incidence in chondrosarcoma is still unknown. This study characterized effect of leptin on production of VEGF in human chondrosarcoma, which is

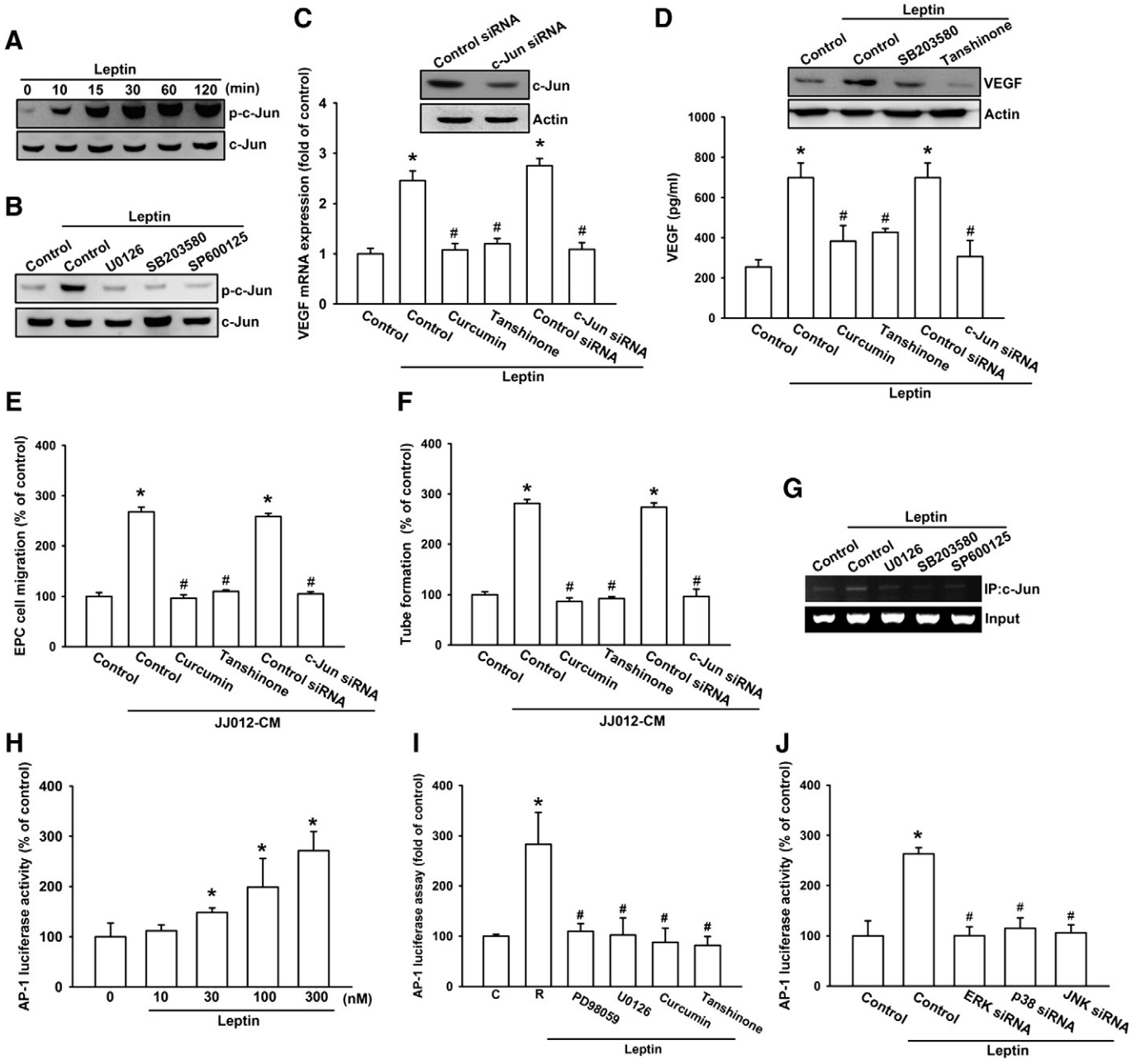

Fig. 4. MAPK pathways are involved in leptin-mediated AP-1 activation and subsequently promoting VEGF production and angiogenesis. (A) JJ012 cells were treated with leptin for indicated time intervals, c-Jun phosphorylation determined by Western blot. (B) JJ012 cells were pre-treated with U0126, SB203580, or SP600125 for 30 min followed by 24 h stimulation with leptin, phosphorylation of c-Jun examined by Western blot. (C) JJ012 cells were pre-treated with curcumin or tanshinone for 30 min or transfected with c-Jun siRNA for 24 h, followed by 24 h stimulation with leptin. VEGF mRNAs were monitored by q-PCR (bottom) (n = 5). Knockdown efficiency of c-Jun was verified by Western blot (top). (D) VEGF protein expression was tested by ELISA (left) and Western blot (right) (n = 4). (E–F) CM were collected, then applied to EPCs for 24 h. Effect of CM on cell migration and capillary-like structure formation in EPCs was rated by Transwell (n = 3) and tube formation assay (n = 6). (G) JJ012 cells were pretreated with U0126, SB203580, and SP600125 for 30 min then stimulated with leptin for 2 h. Binding of c-Jun to VEGF promoter was ChIP assayed. Precipitated chromatin (1%) was assayed to verify equal loading (input). (H) JJ012 cells transiently co-transfected for 24 h with AP-1 luciferase and β-galactosidase expression vectors were treated with leptin for 24 h in a dose-dependent manner (n = 5). (I–J) Transient transfected JJ012 cells pretreated with U0126, SB203580, and SP600125 or cotransfected with ERK, p38, and JNK siRNA, followed by 24 h stimulation with leptin. AP-1 luciferase activity was gauged, results normalized to β-galactosidase activity (n = 5). Bars depict means ± SEM. $^{*}P < 0.05$ compared to control; $^{\#}P < 0.05$ compared to leptin-treated group.

responsible for subsequently increased migration and tube formation in endothelial progenitor cells. Results indicated leptin increases VEGF expression through OBRl receptor, MAPKs (p38, ERK, and JNK), and AP-1 signal transduction and contributes to tumor growth and tumor-associated angiogenesis.

Adipose tissue is one primary leptin production site; this hormone also emanates from actively angiogenic placenta and fetal tissue (like heart and bone/cartilage) [38]. It is noteworthy that leptin is likewise co-expressed with VEGF in these angiogenic tissues [23], which suggests leptin promoting neovascularization and modulating the angiogenic activity of VEGF in these tissues. Regarding tumor angiogenesis, however, accumulating evidence reveals leptin secreting vascular endothelial growth factors as well as potentiating migration and tube formation of endothelial cells to shape tumor microenvironment [39]. This hypoxia-sensitive gene is activated by hypoxia-induced factor-1 (HIF-1) interaction with its upstream promoter via conditions often seen in solid tumors [40,41]. Human chondrosarcoma can yield leptin, acting in an autocrine and paracrine manner to expedite angiogenesis, tumor proliferation, migration, and invasion, aggravating malignant potential and jeopardizing patient survival.

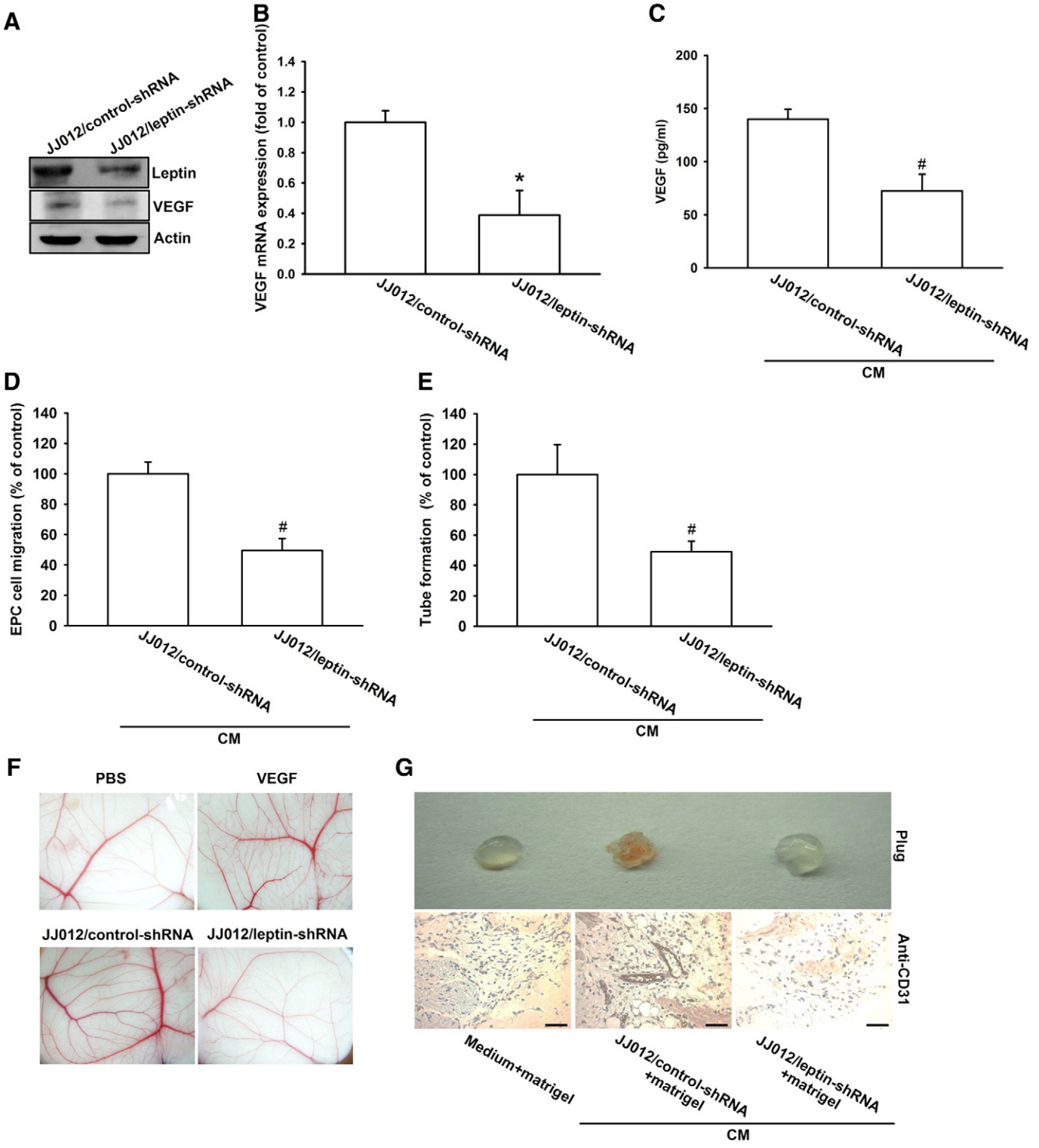

Fig. 5. Knockdown of leptin reduces angiogenetic effects in vitro and in vivo. (A) Western blot of leptin and VEGF expression in JJ012 cells infected with leptin shRNA and control shRNA by lentivirus system. (B–C) mRNA expression of VEGF in JJ012 cells and VEGF protein in CM were tested by q-PCR ($n = 6$) and ELISA ($n = 3$), respectively, after knockdown of leptin by shRNA. (D–E) EPCs were incubated with CM from JJ012/control-shRNA or JJ012/leptin-shRNA for 24 h, cell migration and capillary-like structure formation in EPCs assayed by Transwell ($n = 3$) and tube formation ($n = 6$). (F) Chick embryos incubated with JJ012/control-shRNA CM, PBS, or JJ012/leptin-shRNA CM for 48 h were resected, fixed, then photographed under stereomicroscope (G) Mice were injected subcutaneously with Matrigel mixed with medium, JJ012/control-shRNA CM or JJ012/leptin-shRNA CM. At Day 7, mice were sacrificed, Matrigel plugs excised and processed for light microscopy. Sectioned plugs were immunostained with anti-CD31 antibody, results expressed as mean ± SEM. $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with leptin-treated group.

It has been noted that (1) leptin and/or leptin receptor (OBR) is overexpressed in carcinomatous relative to adjacent normal tissues and (2) leptin/OBR expression positively correlates with degree of malignancy in breast [42], endometrial [43], gastric [44], thyroid [25], and brain cancer [45]. Hence, leptin signaling observably promotes VEGF expression, which synergistically stimulates angiogenesis, inducing cancer growth and progression [20,21,23,46]. Yet to date, there is scant reporting on leptin and VEGF expression profiles in chondrosarcoma;

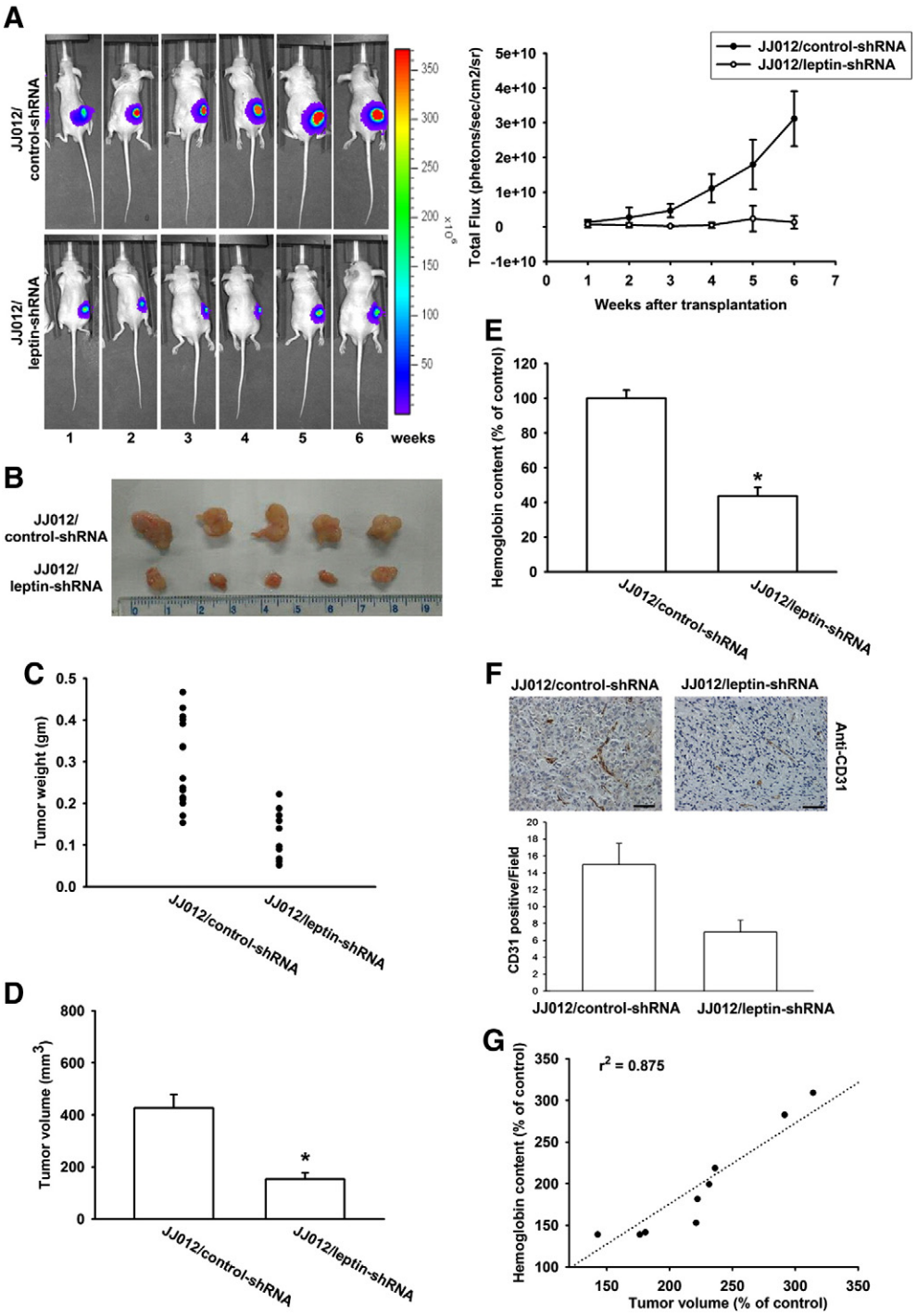

Fig. 6. Depletion of leptin suppresses growth of chondrosarcoma xenografts and angiogenesis in nude mice. (A) Bioluminescent imaging of nude mice subcutaneously inoculated with JJ012/control-shRNA (n = 10) or JJ012/leptin-shRNA (n = 15) stably expressing luciferase. Color scale depicts photon flux (photons/s) emitted from xenografted mice, bioluminescent imaging data quantified at right. (B) Representative tumor growth six weeks after injection (C–E) Tumors measured for weight and volume, hemoglobin quantified. (F) Correlation of hemoglobin with tumor volume. (G) CD31-positive stained microvessels in JJ012/control-shRNA- and JJ012/leptin-shRNA-injected mice tumor sections, results expressed as mean ± SEM. $^{*}P < 0.05$ compared with control.

leptin signaling mechanism to modulate VEGF level is rarely characterized. Leptin exerts its function by binding to transmembrane OBR. Among receptor isoforms, only OBRl contains the full (long) form of OBR (OBRl) that consists of extracellular, transmembrane, and intracellular domain potential, while short OBR isoforms (OBRs) lack major domains recruiting downstream effectors and have diminished or abolished signaling capability [47–50]. MAPK pathways were also involved in leptin-enhanced VEGF expression in breast cancer [21,46]. We hypothesized leptin as regulating VEGF expression through these pathways and found it increasing VEGF production via OBRl receptor/MAPKs/AP-1 signal pathways in human chondrosarcoma to mediate tumor angiogenesis. Leptin has been reported to increase VEGF expression in breast cancer via NF-κB activation [44]. However, NF-κB inhibitor (PDTC; 50 μM) only slightly reversed leptin-increased VEGF expression (Supplementary Fig. S1). Therefore, AP-1 is more important than NF-κB in leptin-mediated VEGF production in chondrosarcoma. In the current study, leptin increased phosphorylation of ERK, p38, and JNK, reaching maximums after 15–30, 30–60, and 10–30 min of treatment, respectively.

Therefore, leptin activated MAPKs at different time period. However, these kinase inhibitors all reduced leptin-mediated c-Jun phosphorylation. Therefore, MAPK signaling are upstream molecules in leptin-induced AP-1 activation. On the other hand, OBRl AS-ODN, ERK, p38, JNK, or c-Jun siRNA all reduced leptin-induced VEGF expression, EPCs migration and tube formation in other chondrosarcoma cell line (SW1353 cells) (Supplementary Fig. S2), indicating same pathways in leptin induced VEGF expression and angiogenesis in chondrosarcoma cells.

Metastasis, particularly to the lungs, is often observed with high-grade chondrosarcomas. Our previous study indicated that leptin can increase αvβ3 integrin expression to promote cell migration in human chondrosarcoma cells through OBR1/IRS-1/PI3K/Akt/NF-κB pathways [51]. However, metastatic colonization cannot be successful without the formation of new blood vessels due to angiogenesis is essential for metastasized tumors to receive essential oxygen and nutrients. Thus, the data that are currently available highlight a crucial role for leptin in regulating VEGF expression, which potentiates migration and tube formation of endothelial cells to change tumor microenvironment affecting the metastatic ability of cancer cells.

5. Conclusion

We found both leptin and VEGF highly expressed in chondrosarcoma tissues, positively correlating with histopathological grade. Results demonstrated that leptin increases VEGF production by activating OBRl receptor and MAPKs (p38, ERK, and JNK), which in turn enhances binding of AP-1 transcription factor to VEGF promoter, transactivating VEGF expression to promote angiogenesis and tumor growth. Findings lend insight into mechanisms underlying leptin pathogenesis, knowledge utilized translationally for novel treatments of chondrosarcoma.

Conflict of interest

The authors declare that no competing interests exist.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.09.012.

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