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Hypoxia regulates angeogenic-osteogenic coupling process *via* up-regulating IL-6 and IL-8 in human osteoblastic cells through hypoxia-inducible factor-1α pathway

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ABSTRACT

Inappropriate angiogenesis and osteogenesis are considered as the crucial factors of osteoporotic fracture. Hypoxia is a primary driving force for regulating the angiogenic-osteogenic coupling process. Our recent results indicated that hypoxia could improve angiogenesis as well as differentiation and activity of osteoblastic cells *via* up-regulating VEGF through HIF-1α pathway. Here we demonstrated that in human osteoblastic MG-63, U2-OS and Saos-2 cells, besides VEGF, the other two pro-angiogenic factors IL-6 and IL-8 were also up-regulated by hypoxia and $CoCl_2$ (a mimic of hypoxia). Mechanism studies indicated overexpression of HIF-1α (generated from transfection with a plasmid encoding sense HIF-1α) markedly increased the levels of IL-6 and IL-8 in osteoblastic cells. Furthermore, a luciferase reporter assay was performed using the reporter vector containing the IL-6 or IL-8 promoter sequence to illustrate observably increased activity of hypoxia-induced IL-6 and IL-8 promoter caused by overexpression of HIF-1α. Additionally, chromatin immune-precipitation analysis showed hypoxia increased the DNA binding ability of HIF-1α to IL-6 or IL-8 promoter. Analysis *in vitro* by MTT test and Boyden chamber assay showed exogenous IL-6 and IL-8 (a relatively short period of treatment with recombinant IL-6 or IL-8 equivalent to the autocrine levels) could significantly promote the proliferation of human osteoblastic, endothelial and monocytic cells, as well as the migration of human endothelial cells. Taken together, these results indicate that IL-6 and IL-8 in osteoblastic cells may also contribute to the angiogenic-osteogenic coupling process *via* HIF-1α pathway. Besides VEGF, IL-6- or IL-8-targeted adjunctive therapy maybe a new strategy to improve the treatment of osteoporosis.

1. Introduction

Osteoporosis is a systemic skeletal disorder associated with micro-architectural deterioration as well as loss of bone mass and density, resulting in 8.9 million fractures worldwide each year [1–3]. Among various causes of delayed or persistent nonunion healing, inappropriate angiogenesis is considered as a crucial factor. Blood vessels carry oxygen and nutrients to the bone and play an important role in bone formation and remodeling by mediating the interactions among osteoblasts, osteocytes, osteoclasts, and vascular cells. Therefore, the processes of angiogenesis and osteogenesis are coupled spatially and temporally in bone formation [4,5]. So far, the nature of the cellular and molecular mechanisms responsible for coupling angiogenesis and osteogenesis remains poorly understood, but a primary driving force is tissue hypoxia.

Hypoxia-inducible factor-1α (HIF-1α) is a master mediator of

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cellular response to hypoxia. In recent years, therapeutic manipulation of HIF-1α pathway for treating bone metabolic disorders such as osteoporosis, is of interests to the orthopaedic researchers [6,7]. Under hypoxic conditions, HIF-1α is stabilized, translocated to the nucleus, and forms the active HIF-1 heterodimer with HIF-1β. Subsequently, the active HIF-1 binds to hypoxia response element (HRE) in the DNA and then regulates the expression of target genes, such as vascular endothelial growth factor (VEGF) [8–10]. Researches on both intramembranous and endochondral bone formation have implicated that VEGF is a critical regulator of angiogenesis and a fundamental mediator of osteogenic response in bone healing [11,12]. Recent work from our laboratory and others have shown that activation of HIF-1α signaling pathway and its downstream target VEGF could result in angiogenesis, and has direct effects on differentiation and activity of osteoblasts [13–15].

Hypoxia could mediate a broad range of hypoxia responsive element genes which are important in regeneration *via* the HIF pathway. To date, more than 100 putative HIF target genes have been identified [16]. In addition to VEGF, there are some other factors involving in angiogenesis. Reports have shown that IL-6 and IL-8, the two multifunctional cytokines that have central roles in the regulation of inflammatory and immune responses, act on neovascularization in several cancers and worsen cancer prognosis [17–20]. Furthermore, they also have direct effects on endothelial cell proliferation and migration [21,22]. IL-6 and IL-8 are produced by a variety of cells, primarily including monocytes, macrophages, several tumour cells, as well as the main bone-forming cells, osteoblasts [23–25]. Therefore, it is worth exploring whether IL-6 and IL-8 play an important role during the process of bone formation and remodeling. However, relatively less is known about the truth at present.

Thus, in the present study, we used human osteoblastic MG-63, U2-OS and Saos-2 cells as *in vitro* models to investigate the effects and the underlying mechanisms of hypoxia on IL-6 and IL-8 expression *via* HIF-1α pathway. Moreover, we employed human umbilical vein endothelial EA.hy926 cells and human acute monocytic leukemia RAW264.7 cells to preliminarily assess the effects of IL-6 and IL-8 on cell proliferation and migration *in vitro*. Here we show that hypoxia could induce IL-6 and IL-8 secretion in human osteoblastic cells and that HIF-1α mediates hypoxia-induced IL-6 and IL-8 expression by directly binding the corresponding promoters. Thus, IL-6 and IL-8, in a bone microenvironment, may represent the potential therapeutic targets for the treatment of osteoporotic fracture.

2. Materials and methods

2.1. Cell culture and treatment

Human osteoblastic MG-63, U2-OS and Saos-2 cells, human umbilical vein endothelial EA.hy926 cells, and human acute monocytic leukemia RAW264.7 cells were purchased from American Type Culture Collection (ATCC, USA). MG-63, EA.hy926 and RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, USA). U2-OS cells were grown in RPMI-1640 medium (Life Technologies, USA). Saos-2 cells were cultured in McCoy's 5A medium (Life Technologies, USA). All cells were cultured in respective medium supplemented with 10% fetal bovine serum (FBS) (Life Technologies), 100 IU/mL penicillin and 100 μg/mL streptomycin (all from Sigma, USA) and kept at 37 °C in an atmosphere of 5% CO_2. For hypoxia culture, MG-63, U2-OS and Saos-2 cells were treated with cobalt chloride ($CoCl_2$) or incubated in a hypoxia incubator of 5%CO_2, 1% oxygen and nitrogen for 24 h.

2.2. Cell transfection

To generate MG-63 stable cell clones with enhanced endogenous HIF-1α expression, pCMVh-HA-ssHIF-1α and the control pCMVh-HA vectors, gifts from Dr. Andrew L Kung (Dana-Farber Cancer Institute, USA), were used. MG-63 cells were transfected with pCMVh-HA-ssHIF-1α or pCMVh-HA vector by Lipofectamine™ 2000 (Invitrogen, San Diego, CA). Selection for the neomycin gene was initiated 48 h after transfection by adding 300 μg/ml of G418 (Invitrogen, USA) to the supplemented culture medium. This selection medium was changed every 2 days for 4 weeks, until all non-transfected cells died. Resistant cell clones were isolated and the efficiency of transfection was detected by Western blotting assay. The optimization cell clones were chosen to expand for further tests.

Additionally, U2-OS and Saos-2 cells were transiently transfected with pCMVh-HA-ssHIF-1α vector or pCMVh-HA control vector by Lipofectamine™ 2000. The transfectants were used for the following tests within 72 h after transfection.

2.3. Cell viability assay

Cell viability was measured by MTT assay. To examine the effect of hypoxia on cell growth, U2-OS and Saos-2 cells were seeded in 96-well plates at 4×10^3 cells per well overnight, then cultured in 1% O_2 or treated with 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1.0, 1.2 mM $CoCl_2$ for 24 h. To detect the effects of IL-6, IL-8, or VEGF on cell viability, MG-63, EA.hy926 and RAW264.7 cells were seeded in 96-well plates at 4×10^3 cells per well and then treated with 0.01, 0.1, 1, 10 ng/ml IL-6, IL-8, or VEGF for 24 h. After treatments, cells were incubated with MTT solution (0.5 mg/ml, Sigma) for additional 4 h at 37 °C in the dark. After removing the supernatant, formazan crystals formed were dissolved in 100 μL DMSO and the absorbance was measured at 490 nm using ELISA microplate reader (Thermo Fisher). Data represent the average absorbance of six wells from one experiment. The percentage of surviving cells was estimated by dividing the A490 nm values of the treated cells by the A490 nm values of the control cells. The experiment was repeated thrice.

2.4. Semi-quantitative RT-PCR

Total RNA was isolated from cells with TRIzol (Invitrogen) according to the manufacturer's instructions. Primer sequences were designed by Vector NTI 8 software and were synthesized by TaKaRa Biotechnology Co., Ltd (Dalian, China). One Step RNA PCR Kit (AMV) (TaKaRa Biotechnology Co., Ltd.) was used to preform RT-PCR. PCR products were subjected to electrophoresis on a 1.5% agarose gel and were analyzed with Quantity One version 4.5.6 software (Bio-Rad, Hercules, CA, USA). The results were normalized against β-actin and were presented as the ratio of target mRNA to β-actin. The primer sequences were as follows: for IL-6, 5′-CCCCCAGGAGAAGATTC CAA-3′(forward) and 5′-CGCAGAATGAGATGAGT

TGT-3′(reverse), for IL-8, 5′-AACATGACTTCCAAGCTGG CCG-3′(forward) and 5′-CAGTTTTCCTTGGGGTCCAGAC-3′ (reverse), for VEGF, 5′-AGGAGGGCAGA

ATCATCACG-3′(forward) and 5′- CAAGGCCCACAGGGATTT TCT-3′(reverse), for β-actin, 5′- TGGAATCCTGTGGCATCCATGA AAC-3′(forward) and 5′-TAAAAC

GCAGCTCAGTAACAGTCC-3′ (reverse).

2.5. Enzyme-linked immunosorbent assay (ELISA)

To evaluate the *in vitro* production of IL-6, IL-8 and VEGF by human osteoblastic cells, 1×10^5 cells were seeded in 24-well plates overnight, and then incubated in an ordinary incubator or a hypoxia incubator of 1%O_2 or treated with $CoCl_2$ for 24 h. After treatment, the supernatants were collected and clarified by centrifugation. The levels of IL-6, IL-8 and VEGF were measured using ELISA kits (Shanghai Senxiong Technologies, China) according to the manufacturer's instructions.

2.6. Western blotting assay

Total protein extraction from human osteoblastic cells was extracted with 1× RIPA lysis buffer plus complete protease inhibitor (Ruentex Biosciences, China). The protein concentrations were quantified using Protein Assay Reagents (Beyotime, China). Equal amounts of proteins were separated by 10–12% SDS-PAGE (Ruentex Biosciences, China), transferred onto PVDF membrane and incubated with specific primary antibodies (anti-HIF-1α at 1:1000 dilutions; anti-β-actin at 1:2500 dilutions) at 4 °C overnight. The membranes were further probed with respective secondary antibodies, and scanned by Odyssey®CLx equipment (LI-COR Biosciences) to detect the bands. In particular, proteins of interest and the control were detected on the same membrane, and the density of the bands was quantified by Odyssey software 3.0 (LI-COR Biosciences).

2.7. Plasmid transfection and luciferase reporter assay

Transfection was performed using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. MG-63 cells were cultured in 12-well plates until 90–95% confluence, followed by transfection. The cells were transfected with 4 μg DNA containing a luciferase reporter gene linked to the proximal region of the IL-6 promoter pGL2-IL-6p-luc (1200 bp) or the IL-8 promoter pGL2-IL-8p-luc (801 bp) along with 2 μg DNA containing pCMVh-HA-ssHIF-1α or pCMVh-HA expression vector, and 2 μg SV40-β gal DNA which was included to evaluate transfection efficiency. After 24 h, the cells were incubated in an ordinary incubator or a hypoxia incubator of 1%O_2 or treated with 0.5 mM $CoCl_2$ for 24 h. Of particular note was that the PMA (200 nM final concentration, Sigma) should be used for MG-63 cells transfected with pGL2-IL-6p-luc vector. Then, the cells were lysed, and luciferase activity was measured using the Luciferase Assay System (Promega, Madison, WI) and recorded using a Thermo Fluoroskan Ascent FL microplate fluorometer and luminometer (Thermo Electron Corporation). Additionally, β-gal activity was detected using ONPG substrate (Sigma) and measured at 420 nm using an ELISA micro plate reader. The luciferase activity levels were normalized to the β-gal activity levels and presented as the mean relative luciferase activity of three independent experiments.

2.8. Chromatin immunoprecipitation (ChIP) assay

One million of MG-63cells were incubated under normoxic or hypoxia conditions or treated with 0.5 mM $CoCl_2$ for 24 h. And then ChIP analysis was performed. Briefly, cells were washed with PBS, cross-linked with 1% formaldehyde at room temperature for 10 min, lysed and sonicated to obtain DNA fragments. After preclearing with blocked protein G agarose, the supernatant was immunoprecipitated by adding anti-HIF-1α antibody (6 μg) (Rabbit polyclonal antibody for ChIP assay, Abcam, USA) or an equivalent concentration of rabbit IgG, followed by incubation at 4 °C overnight. After several washings, the protein was digested with proteinase K (10 g/ml) at 45 °C for 2 h, cross-linking between DNA and protein was reversed and DNA was eluted from the immune complexes. A total of 5 μL of DNA sample was subjected to PCR amplification using the following primers corresponding to the promoter region of human IL-6 (−344 to −117 , 228 bp), IL-8 (−420 to −21, 400 bp) and VEGF (−1041 to −750, 292 bp). The primer sequences were as follows: for IL-6, 5′-GCGCTAGCCTCAATGACGACCT AAG-3′ (forward) and 5′-GAGCCTCAGACATCTCCAGTCCTAT-3′ (reverse), for IL-8, 5′–GATTGGCTGGCTTATCTTC-3′ (forward) and 5′-CATCACCCTACTAGAGAAC-3′ (reverse), for VEGF, 5′-CAGGAACAA GGGCCTCTGTCT-3′ (forward) and 5′-TGTCCCTCTGACAATGTGCC ATC-3′ (reverse).

2.9. Transwell migration assay

Human umbilical vein endothelial EA.hy926 cells' migration was evaluated using a modified Boyden chamber assay (Corning Life Sciences, Netherlands). Briefly, cells were digested using trypsin/EDTA, harvested by centrifugation, re-suspended in 1640 media containing 1% FBS and plated into the matrigel coated upper chamber. Conditioned 1640 media containing 0.01, 0.1, 1 ng/ml IL-6, or IL-8, or VEGF, or an equivalent volume vehicle control were placed in the lower chamber. After 48 h incubation at 37 °C, the lower side of the filter was washed with PBS thrice and fixed with 4% paraformaldehyde solution. After 15mins, cells were dyed with crystal violet and photographed using a microscope. The number of cells in five 200× microscopic fields was averaged and used as the assessment criteria for cell migration ability.

2.10. Statistical analysis

The data were obtained from three independent experiments and were presented as mean ± standard deviation (SD) and processed with the statistics software SPSS 13.0. Multiple comparisons were performed using one-way analysis of variance (ANOVA) with Fisher's protected least significant difference method for post hoc analysis. Statistical differences between two groups were evaluated using the Student's *t* test. All statistical tests were two-sided. *P* value of < 0.05 was considered statistically.

3. Results

3.1. Up-regulation of IL-6 and IL-8 induced by hypoxia is exhibited broadly in human osteoblastic cells

Multiple types of cells, including human osteoblasts, could produce IL-6 and IL-8. Results of our study indicate that the tress of hypoxia may significantly augment the levels of IL-6 and IL-8 in human osteoblastic cell lines. Firstly, we demonstrated the mimic effects of $CoCl_2$ similar to 1%O_2 on cell lines by MTT test and western blotting assay. As shown in Fig. 1, $CoCl_2$ treatment for 24 h significantly inhibited cell proliferation in a dose-dependent manner ($p < 0.01$) and the inhibitory effect similar to 1%O_2 occurred at the dose of 0.5 mM for MG-63 cells (A), 0.4 mM for U2-OS cells (B), and 0.3 mM for Saos-2 cells (C), respectively. The corresponding results of HIF-1α protein level by western blotting assay confirmed the above assumptions of $CoCl_2$.

Secondly, we specified the effects of hypoxia on IL-6 and IL-8 expression in human osteoblastic cells. As shown in Fig. 2A and B, treatment of MG-63 with hypoxia (1%O_2) or 0.5 mM $CoCl_2$ for 24 h resulted in (3.39 ± 0.23)-fold or (3.00 ± 0.20)-fold increase in mRNA expression of IL-6, (5.70 ± 0.11)-fold or (5.30 ± 0.21)-fold increase in mRNA expression of IL-8, and (2.59 ± 0.28)-fold or (2.45 ± 0.19)-fold increase in mRNA expression of VEGF, respectively. Similarly, the protein levels of IL-6, IL-8 and VEGF increased by several folds compared with normoxia group, as determined by western blotting assay (Fig. 2C–E).

To determine whether the up-regulation of IL-6 and IL-8 by hypoxia was specific for MG-63 cells or not, we next studied the other two human osteoblastic cells, U2-OS and Saos-2. As shown in Fig. 3, in response to 1%O_2 or 0.4 mM $CoCl_2$, U2-OS cells showed robust increase above basal mRNA and protein expression of IL-6, IL-8 and VEGF. As for Saos-2 cells, there was a little different manifestation on the IL-6 protein expression. As shown in Fig. 4, the mRNA and protein levels of IL-8 and VEGF in Saos-2 were significantly promoted by hypoxia compared with the normoxia group. However, there was no basal protein expression of IL-6 tested in supernatant by ELISA, whatever under normoxia or hypoxia conditions. Taken together, although no IL-6 protein expression was detected in Saos-2 cells, the above results basically indicate that the up-regulation of IL-6 and IL-8 induced by hypoxia is exhibited broadly

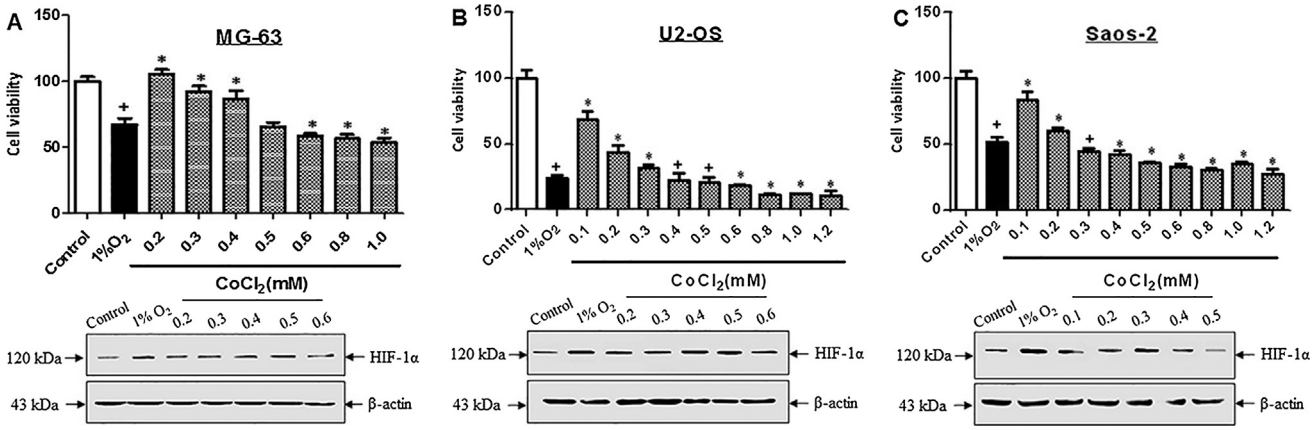

Fig. 1. The mimic effects of $CoCl_2$ similar to $1\%O_2$ occur at different dose for human osteoblastic cells. Human osteoblastic MG-63(A), U2-OS(B) and Saos-2(C) cells were treated with $1\%O_2$ or increased concentrations of $CoCl_2$ (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1.0 or 1.2 mM) for 24 h. After treatment, cell viability was detected by MTT assay and protein lysates from cells were prepared as well as analyzed by western blotting assay. Representative result from three independent experiments was showed. Data represent mean ± SD from three independent experiments. *: $P < 0.01$, $^{+}$: $P < 0.001$ compared with control group.

in human osteoblastic cells.

3.2. Up-regulation of IL-6 and IL-8 is associated with overexpression of HIF-1α in human osteoblastic cells

HIF-1α is the best characterized marker for hypoxia and is sensitively accumulated under hypoxia [26]. Results were reported that the HIF-1α pathway is a critical mediator of neoangiogenesis, which is required for skeletal regeneration [11,12]. The classical genomic functions of HIF-1α are initiated by its binding to the nuclear HIF-1β, followed by dimerization and subsequent binding to HREs [27]. As a consequence, we hypothesized that the up-regulation of IL-6 and IL-8 was associated with the active HIF-1α in osteoblastic cells. MG-63 cells were stably transfected with pCMVh-HA-ssHIF-1α (i.e., sense HIF-1α vector) and the empty vector pCMVh-HA by Lipofectamine™ 2000 (Invitrogen, San Diego, CA), respectively. As shown in Fig. 5A, compared with the empty vehicle control, the enhanced protein levels of HIF-1α in 11 chosen stable clones were 0, 10.45, 6.64, 0, 0, 3.63, 3.92, 6.41, 0, 0 and 3.94 times, respectively. The #3 stable clone exhibited maximum increase of HIF-1α (i.e., MG-63/ssHIF-1α) and the corresponding control (i.e., MG-63/pCMVh-HA) were chosen for subsequent studies. As illustrated in Fig. 5B-E, compared with the parent control cells, MG-63/ssHIF-1α cells had a significant increase in the mRNA and protein expression of IL-6, IL-8 and VEGF. Similar results were observed

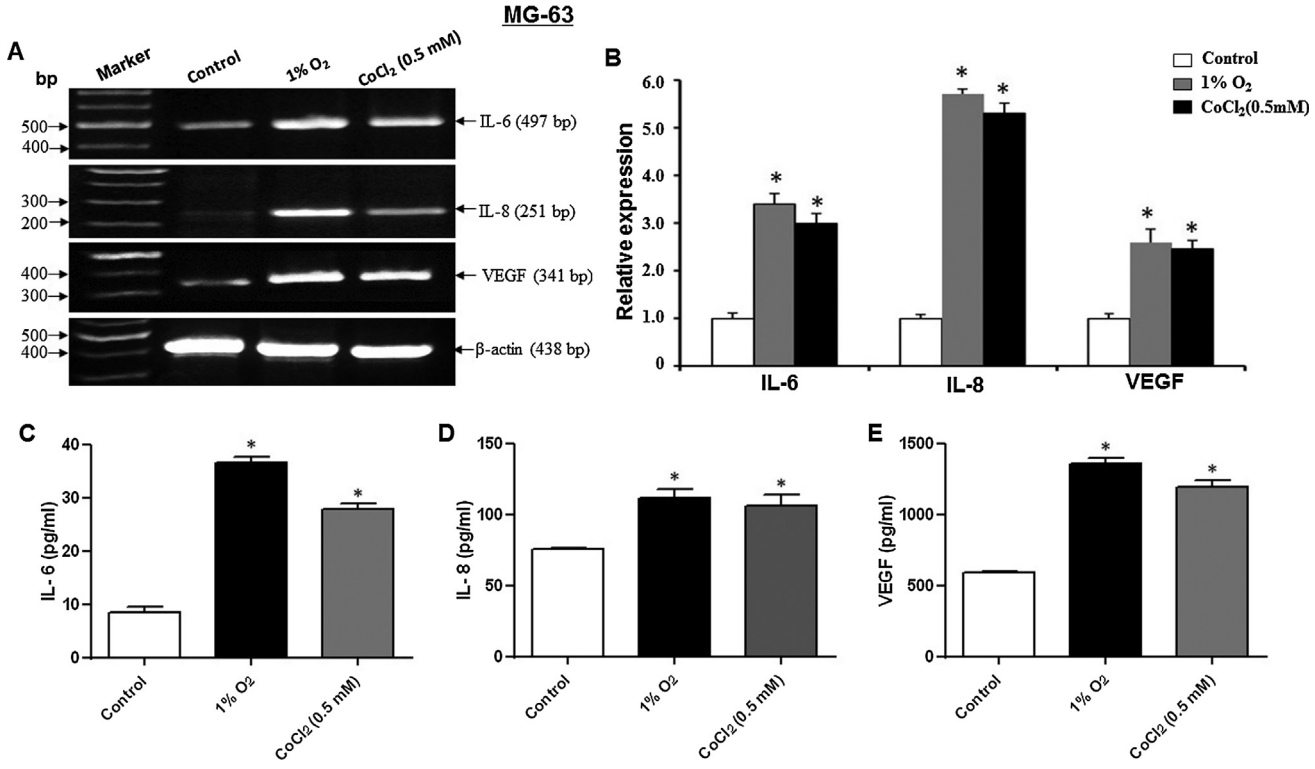

Fig. 2. Up-regulation of IL-6, IL-8 and VEGF by hypoxia in human osteoblastic MG-63 cells. MG-63 cells were treated with $1\%O_2$ or 0.5 mM $CoCl_2$ for 24 h and then followed by subsequent tests. (A-B) Detection of mRNA levels of IL-6, IL-8 and VEGF by semi-quantitative RT-PCR. The target gene expression was normalized using β-actin as control. Representative result from three independent experiments was showed. (C-E) Detection of protein levels of IL-6(C), IL-8(D) and VEGF(E) by ELISA. Data represent means ± SD from three independent experiments. *: $P < 0.01$ compared with control group.

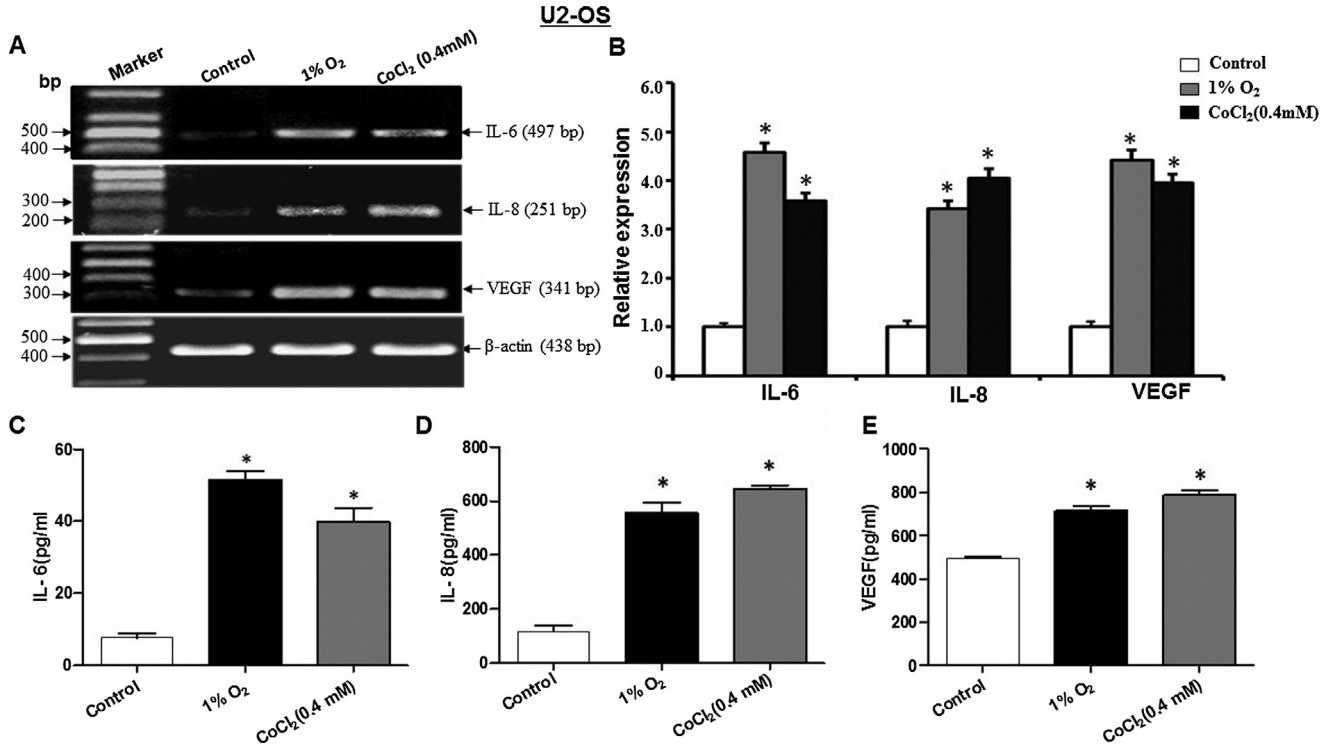

Fig. 3. Up-regulation of IL-6, IL-8 and VEGF by hypoxia in human osteoblastic U2-OS cells. U2-OS cells were treated with 1%O_2 or 0.4 mM $CoCl_2$ for 24 h and then followed by subsequent tests. (A-B) Detection of mRNA levels of IL-6, IL-8 and VEGF by semi-quantitative RT-PCR. Representative result from three independent experiments was showed. (C-E) Detection of protein levels of IL-6(C), IL-8(D) and VEGF(E) by ELISA. Data represent means ± SD from three independent experiments. *: $P < 0.01$ compared with control group.

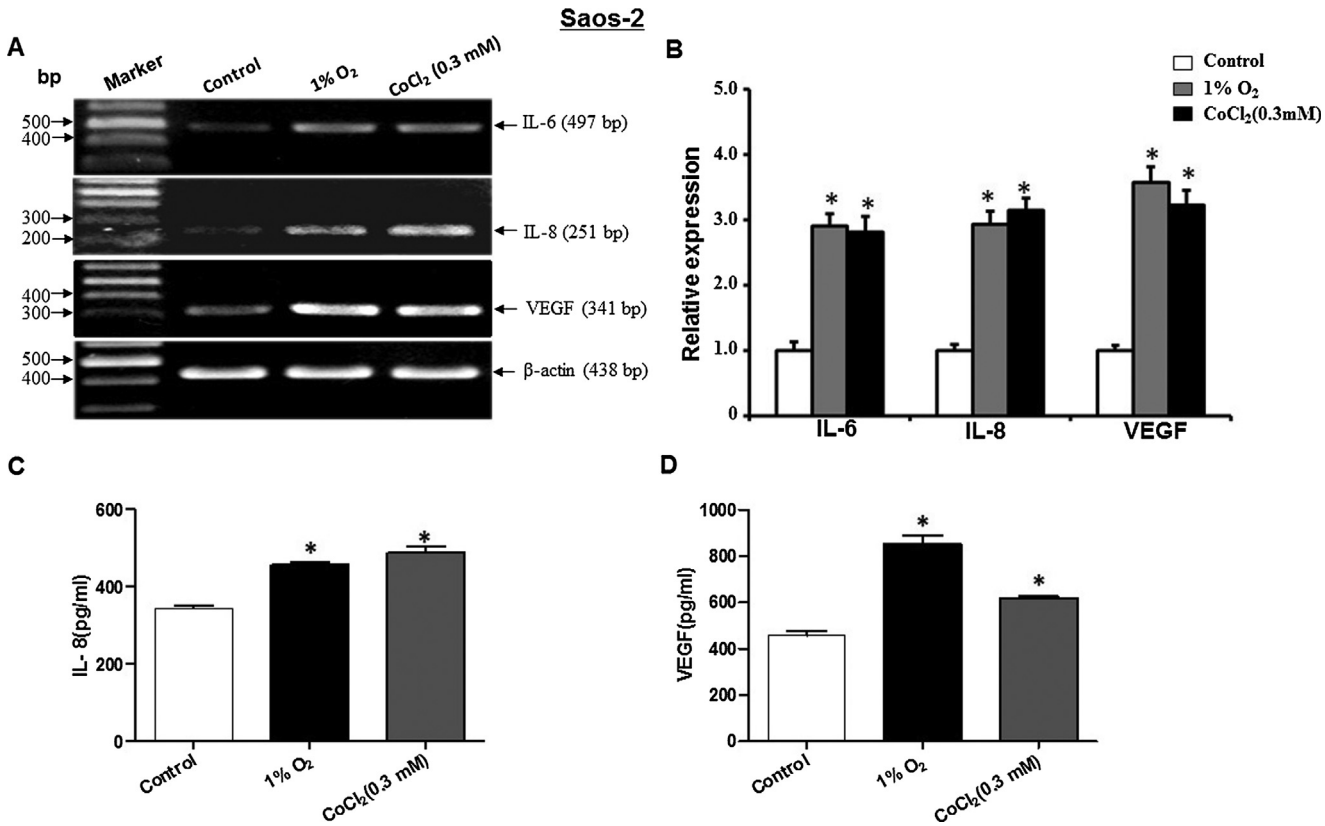

Fig. 4. Up-regulation of IL-6, IL-8 and VEGF by hypoxia in human osteoblastic Saos-2 cells. Saos-2 cells were treated with 1%O_2 or 0.3 mM $CoCl_2$ for 24 h and then followed by subsequent tests. (A-B) Detection of mRNA levels of IL-6, IL-8 and VEGF by semi-quantitative RT-PCR. Representative result from three independent experiments was showed. (C-D) Detection of protein levels of IL-8(C) and VEGF(D) by ELISA. Data represent means ± SD from three independent experiments. *: $P < 0.01$ compared with control group.

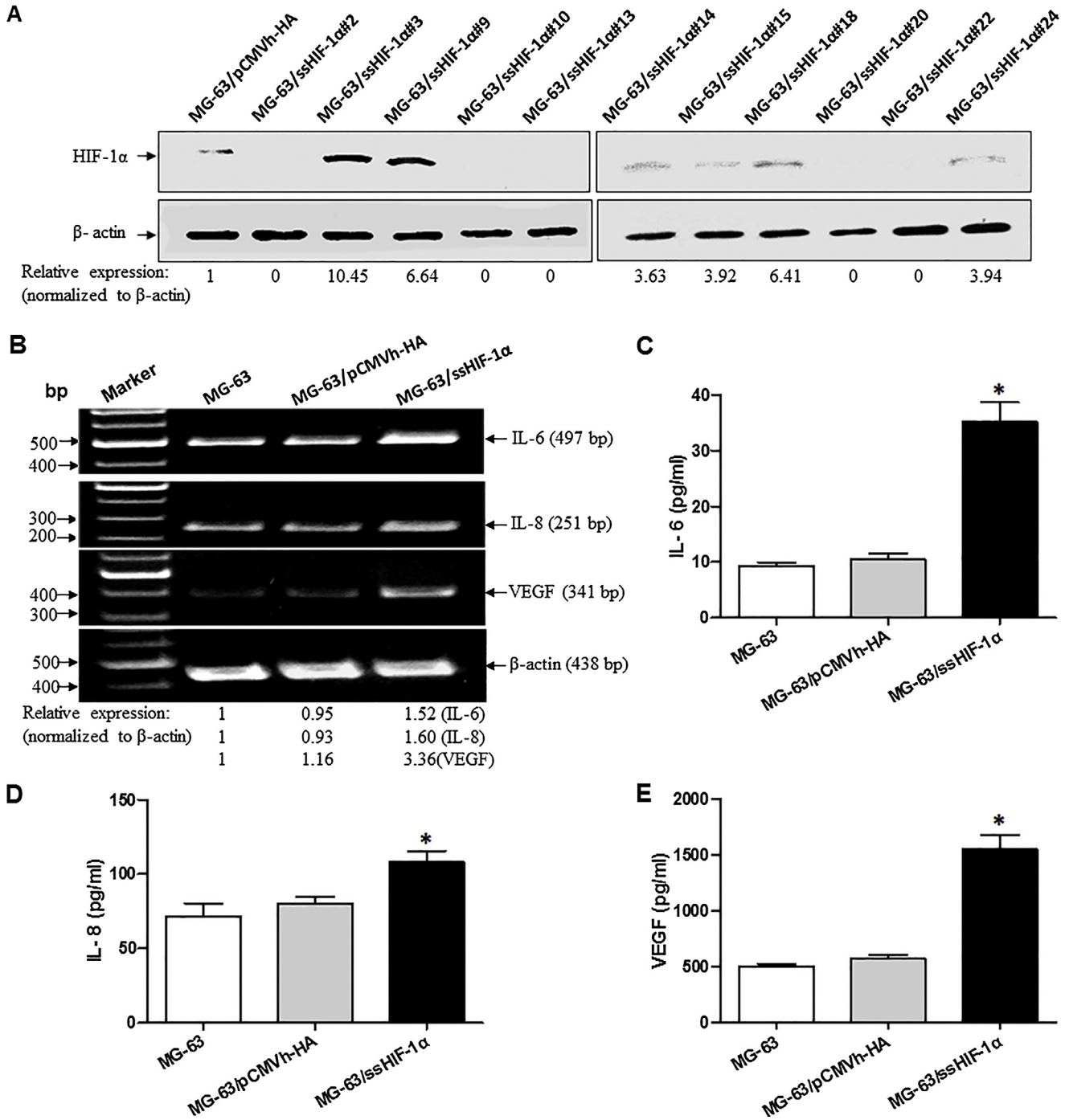

Fig. 5. HIF-1α overexpression promotes IL-6, IL-8 and VEGF levels in human osteoblastic MG-63 cells. (A) MG-63 cells were stably transfected with pCMVh-HA-ssHIF-1α (i.e. sense HIF-1α vector) and the empty vector pCMVh-HA, respectively. Protein lysates were prepared from transfectants and analyzed by western blotting assay. Stable transfectant #3(i.e. MG-63/ssHIF-1α), with maximum up-regulation of HIF-1α, was chosen for subsequent studies. Representative result from three independent experiments was showed. (B) Detection of mRNA levels of IL-6, IL-8 and VEGF in the parent, empty vehicle control and MG-63/ssHIF-1α by semi-quantitative RT-PCR. Representative result from three independent experiments was showed. (C-E) Detection of IL-6(C), IL-8(D) and VEGF(E) protein levels in supernatant derived from the parent, empty vehicle control and MG-63/ssHIF-1α by ELISA. Data represent means ± SD from three independent experiments. *: $P < 0.01$ compared with the parent group.

in other two human osteoblastic U2-OS and Saos-2 cells. As shown in Figs. 6 and 7, after transiently transfected with pCMVh-HA-ssHIF-1α, U2-OS and Saos-2 cells had a significant increase in the expression of IL-6, IL-8 and VEGF. Consistent with the results of Fig. 4, no IL-6 protein expression was observed in Saos-2 cells. These results demonstrated HIF-1α overexpression, induced by hypoxia condition, may promote IL-6 and IL-8 autocrine levels in human osteoblastic cells.

3.3. Transcriptional regulation of IL-6 and IL-8 in human osteoblastic MG-63 cells under hypoxia is mediated via HIF-1α pathway

Analysis of the IL-8 promoter region shows consensus binding sites for HIF-1α, i.e., hypoxia response element (HRE; CGTG) [28]. Since our studies had indicated that HIF-1α overexpression could possibly increase the autocrine levels of IL-6 and IL-8 in osteoblastic cells, we next examined whether the enhanced regulation of IL-6 and IL-8 occurred at

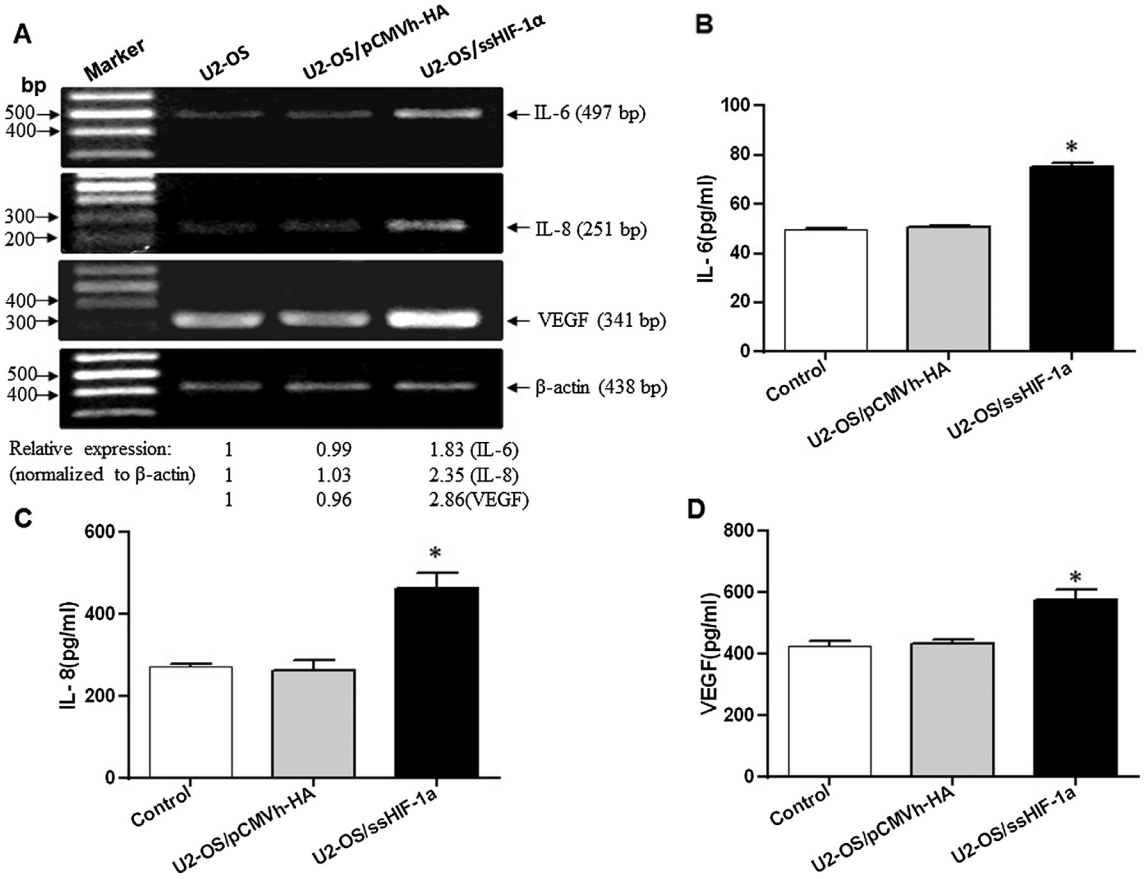

Fig. 6. HIF-1α overexpression promotes IL-6, IL-8 and VEGF levels in human osteoblastic U2-OS cells. (A) U2-OS cells were transiently transfected with pCMVh-HA-ssHIF-1α (i.e. U2-OS/ssHIF-1α) and the empty vector pCMVh-HA, respectively. Protein lysates were prepared from transfectants and analyzed by western blotting assay. (B-E) Detection of mRNA levels (B) as well as protein levels of IL-6(C), IL-8(D) and VEGF(E) in the parent, empty vehicle control and U2-OS/ssHIF-1α by semi-quantitative RT-PCR and ELISA, respectively. Representative result from three independent experiments was showed. Data represent means ± SD from three independent experiments. *: $P < 0.01$ compared with the parent group.

the transcriptional level. MG-63 cells were transfected with the pGL2-IL6p(-1200)-luc that contains the proximal 1200 bp of the IL-6 promoter or pGL2-IL8p(-801)-luc that contains the proximal 801 bp of the IL-8 promoter followed by incubation 1%O_2 or 0.5 mM $CoCl_2$. As shown in Fig. 8, HIF-1α overexpression strongly enhanced hypoxia-induced transcriptional activation of the IL-6 promoter and IL-8 promoter in human osteoblastic MG-63 cells.

3.4. Hypoxia enhances the binding ability of HIF-1α to promoters of IL-6 or IL-8 in human osteoblastic MG-63 cells

To investigate the binding activity of HIF-1α to promoters of IL-6 or IL-8, we next performed ChIP analysis using human osteoblastic MG-63 cells treated with 0.1% O_2 or 0.5 mM $CoCl_2$ for 24 h. As illustrated in Fig. 9, anti-HIF-1α antibodies and normal rabbit IgG were used to immunoprecipitate sheared chromatin, and then PCR amplification was conducted. In MG-63 cells treated with 0.1% O_2 or 0.5 mM $CoCl_2$, IL-6, or IL-8, or VEGF DNA products were detected at a significantly higher level than normoxia condition. This result strongly suggested that in hypoxia condition, after translocated into nucleus, HIF-1α binds not only to the promoter of VEGF, the classical downstream target of HIF-1α, but also to the promoters of IL-6 and IL-8. In combination with the results of Fig. 8, these data on the molecular mechanisms directly confirmed that HIF-1α could bind IL-6 and IL-8 promoters and then enhance the transcription of them in human osteoblastic cells.

3.5. IL-6 and IL-8 expression mediated by hypoxia may play an important role in coupling angiogenesis with osteogenesis during bone development and regeneration

During the osteogenesis-angiogenesis coupling process, osteoblasts, osteoclasts differentiated from monocytes, and vascular cells consist of the main components in the microenvironment of bone formation and repair. Given the exposure of human osteoblastic cells to hypoxia or $CoCl_2$ caused enhanced autocrine IL-6, IL-8 and VEGF, we then investigated the effects of recombinant human IL-6, IL-8, and VEGF on the proliferation as well as migration of osteoblastic, monocytic and endothelial cells. As illustrated in Fig. 10A and B, treatment with 0.01–1 ng/ml IL-6, or IL-8, or VEGF, which was equivalent to the autocrine levels in human osteoblastic cells (Figs. 1, 3 and 4), could markedly promote proliferation of human osteoblastic MG-63 and monocytic RAW264.7 cells. Additionally, augmented endothelial cells' proliferation and enhanced migration abilities were both seen in IL-6, IL-8, or VEGF treated EA.hy926 cells, although VEGF exhibited a stronger stimulation than IL-6 and IL-8 (Fig. 10C and 11). These results indicated that IL-6 and IL-8 were both responsible for the activation of hypoxia on the enhanced bone remodeling in the manner of autocrine and paracrine.

4. Discussion

Inappropriate angiogenesis and osteogenesis have been considered as the crucial causes for delayed or persistent nonunion healing in osteoporosis patients with fractures. Hypoxia, as a strong stimulus for

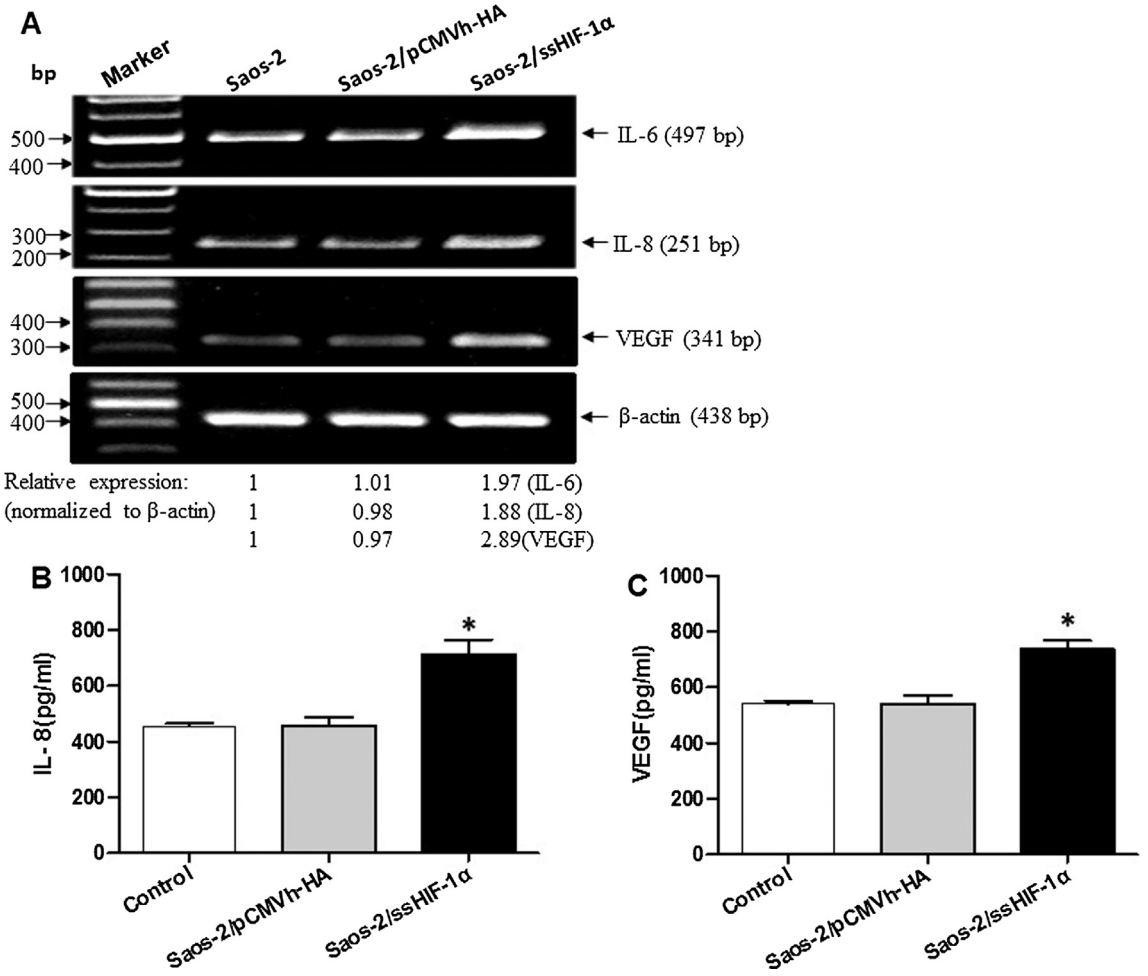

Fig. 7. HIF-1α overexpression promotes IL-8 and VEGF expression in human osteoblastic Saos-2 cells. (A) Saos-2 cells were transiently transfected with pCMVh-HA-ssHIF-1α (i.e. Saos-2/ssHIF-1α) and the empty vector pCMVh-HA, then analyzed the HIF-1α level by western blotting assay. (B-D) Detection of mRNA levels of IL-6, IL-8 and VEGF(B) as well as protein levels of IL-8(C) and VEGF(D) in the parent, empty vehicle control and Saos-2/ssHIF-1α cells by semi-quantitative RT-PCR and ELISA, respectively. Representative result from three independent experiments was showed. Data represent means ± SD from three independent experiments. *: $P < 0.01$ compared with the parent group.

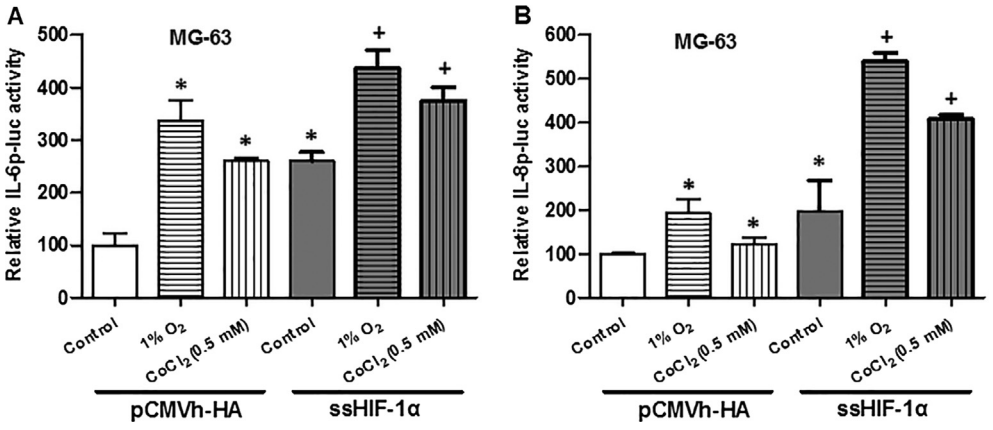

Fig. 8. Transcriptional regulation of IL-6 and IL-8 in human osteoblastic MG-63 cells under hypoxia is mediated by HIF-1α. pGL2-IL-6p (1200 bp) (A) or pGL2-IL-8p (801 bp) (B) and β-galactosidase vectors were co-transfected into MG-63 cells with vectors encoding for sense HIF-1α (ssHIF-1α) or with empty vector (pCMVh-HA), and then cells were treated with 1%O_2 or 0.5 mM $CoCl_2$ for 24 h. After treatment,β-galactosidase and luciferase values were measured, and the luciferase activities were normalized to the β-galactosidase values. Data were presented as the mean normalized luciferase activities of three independent experiments. *: $P < 0.01$, +: $P < 0.001$ compared with the parent group.

stabilizing HIF-1α expression and the followed VEGF production in cells, contributes largely to the process of fracture healing. However, the nature of the cellular and molecular mechanisms responsible for coupling angiogenesis with osteogenesis remains poorly understood. Here, in this paper, we mainly focused on the roles of IL-6 and IL-8 production in human osteoblasts induced by hypoxia or $CoCl_2$ and further explored the corresponding molecular mechanisms. We showed that hypoxia and $CoCl_2$ could induce IL-6 and IL-8 secretion in human osteoblastic cells *via* HIF-1α signaling. Therefore, besides VEGF, IL-6 and IL-8 may also act as the downstream targets of HIF-1α answering to hypoxia stress and play an important role in the improvement of osteoporotic fracture repair.

As the multifunctional chemokines, IL-6 and IL-8 can be secreted by multiple cell types, including monocytes, neutrophils, endothelial and mesothelial cells. With a defining CXC amino acid motif, IL-6 and IL-8 are responsible for recruiting neutrophils, T cells, and basophils during

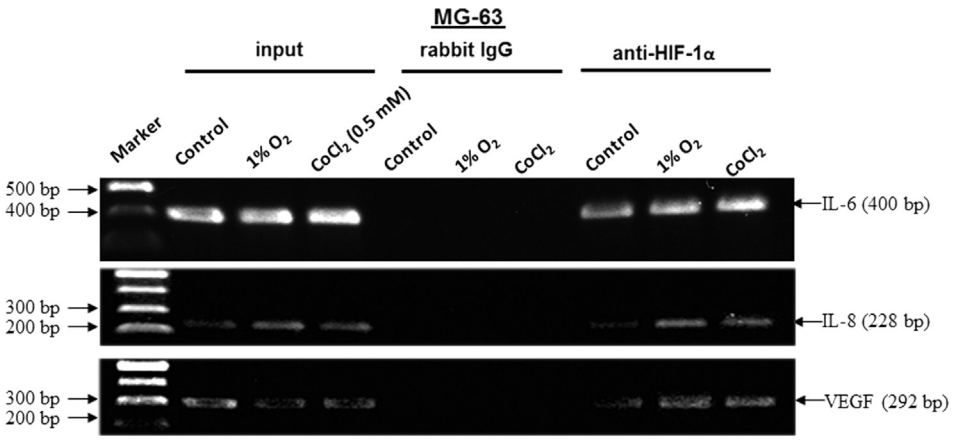

Fig. 9. Hypoxia enhances the binding ability of HIF-1α to promoters of IL-6 or IL-8 in human osteoblastic MG-63 cells. ChIP was performed using control IgG and anti- HIF-1α antibodies to immunoprecipitate sheared chromatin. After digestion with proteinase K, DNA was eluted from the DNA-protein immune complexes and subjected to PCR amplification using the IL-6, IL-8 and VEGF primers. Representative result from three independent experiments was showed.

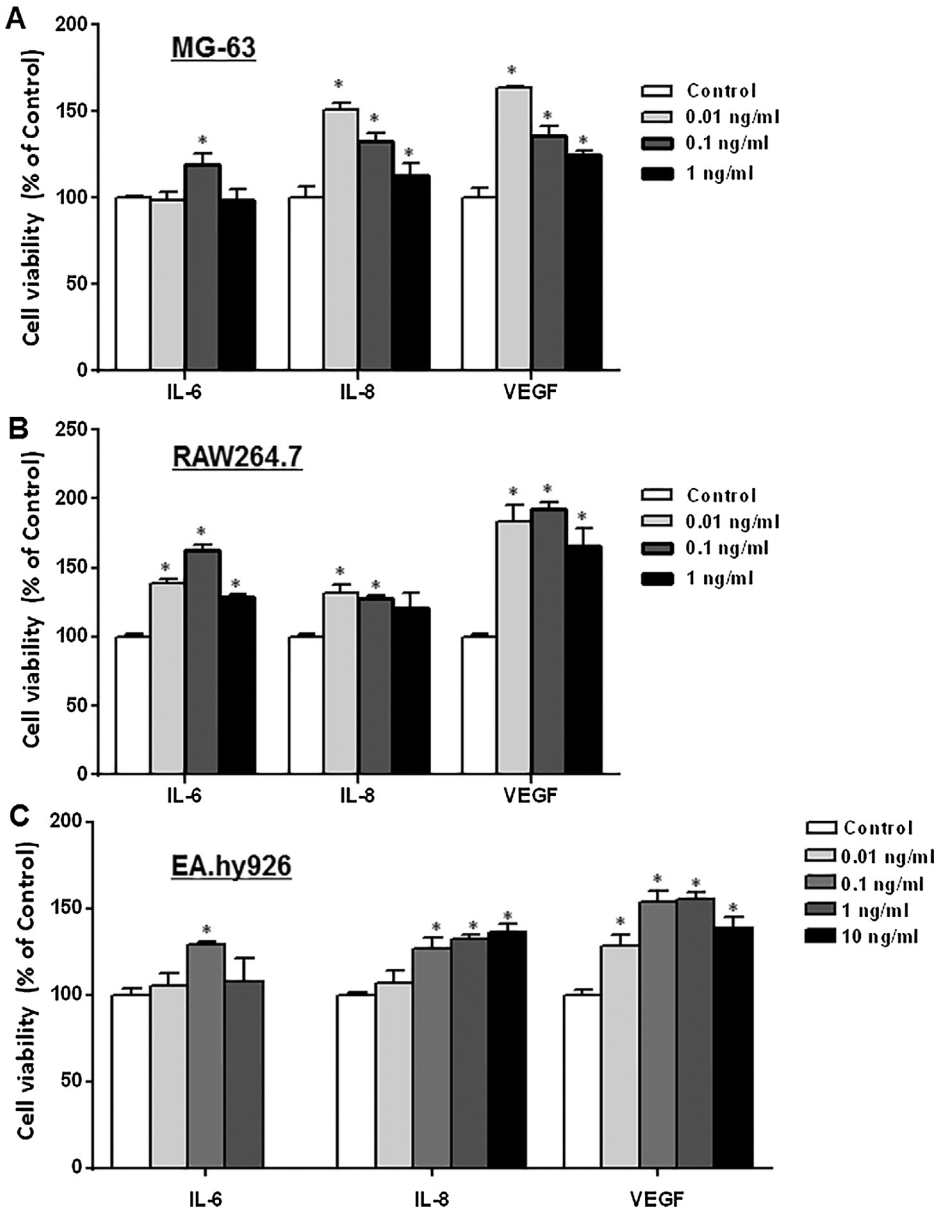

Fig. 10. IL-6, IL-8, and VEGF promote proliferation of osteoblastic, endothelial and monocytic cells. Human osteoblastic MG-63 cells (A), human umbilical vein endothelial EA.hy926 cells (B), and human acute monocytic leukemia RAW264.7 cells (C) were treated with different concentration of IL-6, IL-8 or VEGF for 24 h. After treatment, cell viability was detected by MTT assay. Data represent means ± SD from three independent experiments. *: $P < 0.01$ compared with control group.

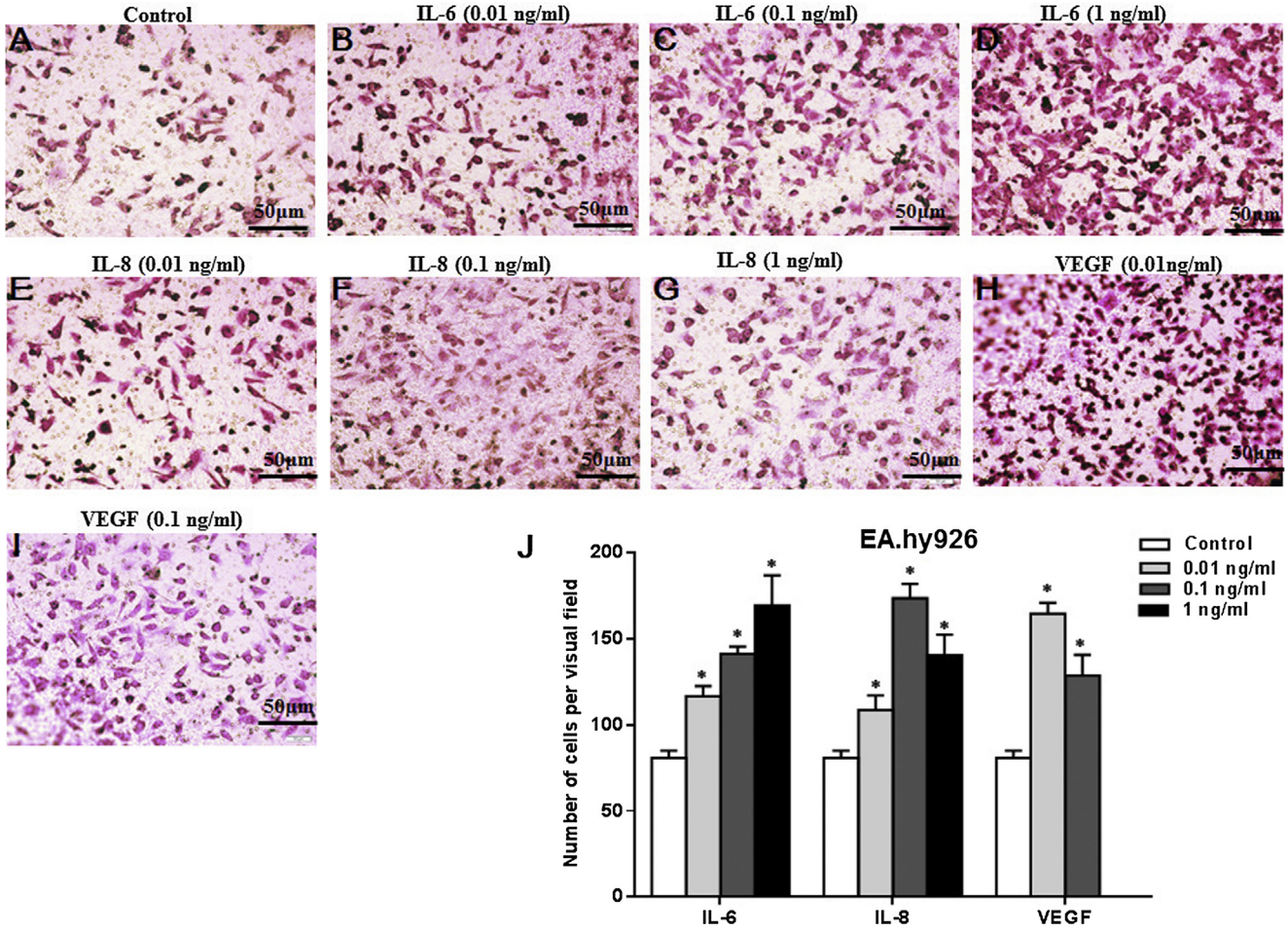

Fig.11. IL-6, IL-8, and VEGF potentiate *in vitro* migration of human umbilical vein endothelial EA.hy926 cells. EA.hy926 cells were treated with vehicle(A) or different concentration of IL-6(B-D), or IL-8(E-G), or VEGF(H-J) for 24 h. After treatment, the *in vitro* migration of cells was photographed at ×200 magnification and measured by determined cell counts that penetrated through Matrigel-coated Transwell chambers (8 μm pore size) (J). Data represent means ± SD from three independent experiments. *: $P < 0.01$ compared with control group.

immune system activation [29]. In addition, IL-6 and IL-8 also act as the directly pro-angiogenic factors to promote angiogenic responses in *in vivo* models and are markedly associated with tumor angiogenesis [29,30]. Previous studies have shown that there are increased levels of IL-6 and IL-8 in sera from patients with osteoporosis [31]. Researches on osteogenesis also provided the description of autocrine IL-6 and IL-8 in osteoblasts [32], which was verified likewise by our studies with three usual human osteoblastic cell lines MG-63, U2-OS and Saos-2. As shown in Figs. 1–4, both mRNA and protein levels of IL-8 in three human osteoblasts could be detected in normoxia condition. As for IL-6, we didn't find the secretion in Saos-2 cells. Importantly, besides VEGF, both IL-6 and IL-8 expression in human osteoblasts could be promoted in response to hypoxia and $CoCl_2$. Given the pro-angiogenic effects by IL-6 and IL-8, we speculated IL-6 and IL-8 might take part in the hypoxia-induced angiogenesis and then promote osteogenesis during the microenvironment of bone formation.

The best characterized response to hypoxia is through the activation of HIF-1, especially the HIF-1α transcription factor. At present, there still is controversy about the correlation between HIF-1α and chemokines of IL-6 and IL-8. Most researchers report that the up-regulation of IL-6 and IL-8 is mediated by HIF-1α signals [18–20,22,23,28,33–37]. The direct proof was found by Kim et al. who proved that the IL-8 promoter region had HRE motif that could bind to HIF-1α and therefore induced the higher expression of IL-8 expression in endothelial cells [28]. Nevertheless, it was also reported that HIF-1 activation inhibited the production of IL-6 and IL-8 in epithelial cells during hypoxia [37,38]. Different tissues and cell types used in previous studies may be one explanation for conflicting results. In our studies, we found endogenous overexpression of HIF-1α could promote hypoxia-induced IL-6 and IL-8 production in human osteoblasts. As illustrated in Fig. 5, transfection with sense HIF-1α vector could markedly enhance IL-6 and IL-8 mRNA and protein expression in MG-63 cells, which was similar to the hypoxia- or $CoCl_2$-induced IL-6 and IL-8 production. Parallel results were found in other two osteoblastic U2-OS and Saos-2 cells after transiently transfected with sense HIF-1α vector (Figs. 6 and 7). Further studies by IL-6- or IL-8- luciferase reporter assay (Fig. 8) suggested that hypoxia-induced up-regulation of IL-6 and IL-8 was mainly mediated by HIF-1α signal and occurred at the transcriptional level. Additionally, ChIP analysis to MG-63 cells (Fig. 9) showed that the affinity between HIF-1α and promoters of IL-6, or IL-8, or VEGF, in hypoxia condition, was significantly increased. The above results are consistent with those of Kim et al. [28]. They found that in the promoter region of IL-8, there existed a functional HIF-1α binding site (CGTG), which in turn provided the basis for the transcription of IL-8 *via* the activation of HIF-1.

Studies on mechanisms of IL-8 promoting angiogenesis show that IL-8 can promote the migration of endothelial cells and vascularization [39]; increase the expression of VEGF and VEGF receptor in tissues [30]. Studies *in vitro* have shown that IL-6 can affect the biological behavior of endothelial progenitor cells and play a crucial role in wound repair as a kind of potential pro-angiogenic factor [21]. Our findings showed the proliferation abilities of human osteogenetic MG-63, monocytic RAW264.7 and endothelial EA.hy926 cells were

significantly increased by treatment of recombinant IL-6 and IL-8, as well as VEGF (Fig. 10). In addition, we also observed the effects of IL-6 and IL-8 on the migration of endothelial EA.hy926 cells in the concentration-dependent manner. Combined with the before-mentioned results on the autocrine levels of IL-6 and IL-8 in human osteoblastic cells in Figs. 1–4, we supposed that osteoblasts in hypoxia condition, may regulate the balance between osteogenesis and angiogenesis by enhanced secreting IL-6 and IL-8.

5. Conclusions

In conclusion, the results from the present study support the concept of the hypoxia-mediated up-regulation of IL-6 and IL-8 in human osteoblastic cells *via* HIF-1α pathway, which may promote osteogenesis by facilitating angiogenesis. Thus, our studies may provide one more avenue, among several approaches, to ameliorate inappropriate fracture and bone healing in patients with osteoporosis.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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