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COMP-Ang1 Accelerates Chondrocyte Maturation by Decreasing HO-1 Expression

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ABSTRACT

Endochondral ossification is essential for new bone formation and remodeling during the distraction stage. Endochondral ossification is attributed to chondrocyte maturation, which is induced by various factors, such as the cellular environment, gene transcription, and growth factor expression. Cartilage oligomeric matrix protein (COMP)-angiopoietin 1 (Ang1) is more soluble, stable, and potent than endogenous Ang1, and COMP-Ang1 treatment has osteogenic and angiogenic effects in an in vivo model of bone fracture healing. Although the osteogenic effects of COMP-Ang1 have been demonstrated, the precise mechanism by which COMP-Ang1 induces chondrocyte maturation and triggers endochondral ossification is not understood. Here, we investigated the possible mechanism by which COMP-Ang1 induces chondrocyte maturation. First, using a WST assay, we found that COMP-Ang1 is nontoxic in rat chondrocytes. Then, we isolated total RNA from COMP-Ang1–treated rat chondrocytes, and analyzed the decrease in chondrogenic gene expression and the increase in osteogenic gene expression using real-time RT-PCR. Gene and protein expression of heme oxygenase-1 (HO-1), which maintains chondrocytes in an immature stage, decreased in a dose-dependent manner upon COMP-Ang1 treatment. To clarify the relationship between HO-1 and COMP-Ang1 in chondrocyte maturation, we used cobalt protoporphyrin IX (CoPP IX), an HO-1 inducer, and tin protoporphyrin IX (SnPP-IX), an HO-1 inhibitor. Treatment with various combinations of CoPP IX, SnPP IX, and COMP-Ang1 confirmed that COMP-Ang1 accelerates chondrocyte maturation by reducing HO-1. In conclusion, our results suggest that COMP-Ang1 accelerates chondrocyte maturation by interacting with HO-1. J. Cell. Biochem. 114: 2513–2521, 2013.

KEY WORDS: COMP-ANG1; BONE FRACTURE; ENDOCHONDRAL OSSIFICATION; CHONDROCYTE MATURATION; HO-1

Chondrocyte maturation is essential to endochondral ossification and precedes callus formation and the establishment of a primary ossification center. Expression of osteogenic genes such as BMP2 and RUNX2 increases greatly during chondrocyte maturation [Drissi et al., 2005]. Likewise, the expression of chondrogenic genes such as collagen type 2 and SOX9 decreases under similar conditions [Tew et al., 2008]. Maturing chondrocytes also have high alkaline phosphatase (ALP) activity [Pullig et al., 2000] and can mineralize the extracellular matrix [Hashimoto et al., 1998]. Taken together, the evidence from these studies strongly supports the possibility that, during endochondral ossification, the phenotype of chondrocytes switches from a permanent cartilage cell to a transient cartilage cell. Therefore, the pathways accelerating chondrocyte maturation may be important for identifying possible osteogenic effects.

Heme oxygenase-1 (HO-1) catalyzes the degradation of heme to iron, carbon monoxide (CO), and biliverdin, and is a novel enzyme

Abbreviations: COMP-Ang1, Cartilage oligomeric matrix protein-angiopoietin 1; CoPP IX, cobalt protoporphyrin IX; DCF-DA, 2′,7′-dichlordihydrofluorescin diacetate; DMEM, Dulbecco's minimal essential medium; HO-1, heme oxygenase-1; ROS, reactive oxidative species; SnPP IX, tin protoporphyrin IX.

Conflicts of interest: None.

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with potent enzymatic activity [Lin et al., 2005]. HO-1 is induced in many cell types as a protective response, and is also expressed in chondrocytes. During chondrocyte maturation, tin protoporphyrin IX (SnPP IX), an HO-1 inhibitor, downregulates HO-1 expression and degrades chondrogenic proteins such as collagen type 2 and SOX9. In contrast, cobalt protoporphyrin IX (CoPP IX), an HO-1 inducer, upregulates HO-1 and chondrogenic protein expression [Guillen et al., 2008]. Therefore, HO-1 affects the regulation of chondrogenic proteins. Based on these results, we hypothesize that HO-1 mediates numerous metabolic processes that may influence chondrocyte maturation.

Cartilage oligomeric matrix protein (COMP)-Angiopoietin 1 (Ang1) is more soluble, stable, and potent than endogenous Ang1. Ang1 is an angiogenic growth factor that mediates the formation of stable and functional vasculature by interacting with Tie2 and Tie1 receptors, which are expressed mainly in vascular endothelial cells, as well as in a subset of monocytes/macrophages [Saharinen et al., 2005]. The structure of Ang1 comprises a carboxy terminal fibrinogen-like domain, a central coiled-coil domain, and a short amino-terminal domain [Cho et al., 2004b]. Large-scale Ang1 production is hindered by aggregation and insolubility resulting from disulfide-linked, higher-order structures. To overcome these problems, the amino terminus of Ang1 was replaced with the short coiled-coil domain of COMP to produce recombinant COMP-Ang1 [Cho et al., 2004b]. Compared with endogenous Ang1, COMP-Ang1 has various benefits, such as maintaining stable vascular enlargement, increasing blood flow [Cho et al., 2004a, 2005], protecting against radiation-induced endothelial cell apoptosis [Cho et al., 2004b], promoting wound healing in diabetic db/db mice [Cho et al., 2006], ameliorating renal fibrosis [Kim et al., 2006a; Lee et al., 2007], preventing hypertension, enhancing insulin sensitivity [Sung et al., 2009], and protecting against cerebral ischemia [Shin et al., 2010].

Recently, COMP-Ang1 was demonstrated to have osteogenic effects, increasing bone mass in vivo and incrementally increasing the expression of the osteogenic genes necessary for osteoblast differentiation [Jeong et al., 2010; Park et al., 2010]. However, the precise mechanism by which COMP-Ang1 affects chondrocyte maturation in relation to endochondral ossification has not been fully elucidated. In this study, we integrated the results of previous studies and investigated whether COMP-Ang1 induces chondrocyte maturation by decreasing HO-1 signaling.

MATERIALS AND METHODS

HARVESTING CHONDROCYTES AND ESTABLISHING CELL CULTURE

Chondrocytes were harvested according to a previously described method with modification [Gomez-Camarillo et al., 2009]. Briefly, chondrocytes were isolated from the articular cartilage of 3-week-old male Sprague–Dawley rats. Animals were sacrificed using an overdose of anesthesia, and cartilage was subsequently removed. The cartilage was cut into thin slices, washed with sterilized phosphate buffered saline (PBS), and soaked in a 5% penicillin–streptomycin solution (Sigma) for 15 min. The cartilage slices were then washed with PBS to remove residual antibiotic solution and digested with 0.02% collagenase type II (Sigma) in Dulbecco's modified Eagle's medium (DMEM, HyClone) for 3 h in a 37°C water bath. The digested cartilage was then collected and centrifuged, and the pellet was resuspended with DMEM and filtered through a 70-μm nylon mesh. The resulting chondrocytes were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin in a 5% CO_2 incubator at 37°C. All experiments were performed when the cells reached confluence and were within the first passage.

IN VITRO CHONDROCYTE MATURATION EXPERIMENTS

We used COMP-Ang1 (Enzo Biochemicals, Farmingdale, NY), SnPP IX, and CoPP IX (Frontier Scientific, Logan, UT) for chondrocyte maturation experiments. A diagram of the cell treatments and experimental analysis scheme is shown in Figure 1. Chondrocyte maturation was evaluated by comparing osteogenic gene expression with chondrogenic gene expression. Specifically, an increase in osteogenic gene expression and a decrease in chondrogenic gene expression suggested chondrocyte maturation.

TOXICITY ASSAY FOR COMP-ANG1-TREATED CHONDROCYTES

The WST assay was used to measure cell toxicity by quantifying formazan absorbance. We used the EZ-Cytox cell viability assay kit (Daeil Lab, Korea). Cells were plated in 96-well plates at a density of 2.0×10^4 cells per well and incubated for 24 h. The cells were subsequently treated with various concentrations (10–500 μg/ml) of COMP-Ang1 and incubated for an additional 24 h. After the incubation period, 10 μl of the kit solution was added to each well and incubated for 3 h at 37°C and 5% CO_2. Cell toxicity was determined by measuring formazan production using a microplate reader at an absorbance of 480 nm. The reference wavelength was 650 nm.

INTRACELLULAR REACTIVE OXYGEN SPECIES ASSAY

The level of intracellular reactive oxygen species (ROS) was quantified by fluorescence using 2′, 7′-dichlordihydrofluorescin diacetate (DCF-DA) (Invitrogen, Carlsbad, CA). Chondrocytes plated in 48-well plates were treated with or without COMP-Ang1 (100 ng/ml) in the absence or presence of 10 μM SnPP IX or CoPP IX and incubated for 6 h. After incubation, the cells were washed with PBS and stained with DCF-DA in PBS for 30 min in the dark. Next, cells were washed with PBS twice

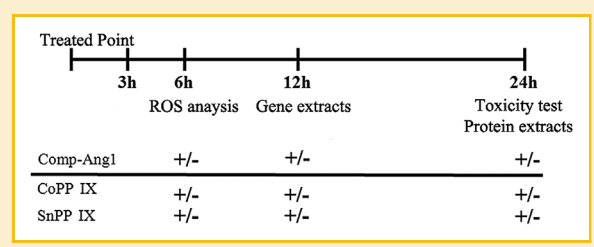

Fig. 1. A schematic diagram of the experimental design. Chondrocytes were treated with CoPP IX, SnPP IX, COMP-Ang1, or a combination and analyzed at several time points. ROS was measured at 6 h. Chondrogenic, osteogenic, and HO-1 gene expression were analyzed at 12 h. COMP-Ang1 toxicity was determined, and immunoblotting was performed at 24 h.

and extracted with 0.1% Tween-20 in PBS (PBS-T) for 10 min at 37°C. Fluorescence was recorded at an excitation wavelength of 490 nm and an emission wavelength of 525 nm.

SMALL INTERFERING RNA TRANSFECTION

Each cell was plated in 6 cm^2 dishes (4×10^5 cells per dish). After 24 h, we used DharmaFECT 1 siRNA Transfection Reagent (Dharmacon, Denver) to transfect the cells with 50 nM Tie2 siRNA or scrambled siRNA oligonucleotides (Dharmacon) according to manufacturer's instructions, as previously reported and validated.

RNA PREPARATION AND REAL-TIME RT-PCR

Total cellular RNA prepared from cells was precipitated with Ribo EX (Geneall, Daejeon, Korea) and dissolved in DEPC-treated distilled water. Total RNA (2 μg) was treated with RNase-free DNase (Invitrogen, Carlsbad, CA) and first strand cDNA was generated using oligoDT primers provided in the Maxime RT PreMix (Intron, Seongnam, Korea) first-strand cDNA synthesis kit according the manufacturer's instructions. Specific primers for each gene (Table I) were designed using Primer Express software (Applied Biosystems, Singapore). Real-time RT-PCR reaction mixtures consisted of 10 μg of reverse-transcribed total RNA, 167 nM forward and reverse primers, and 2× PCR master mix in a final volume of 20 μl. PCR was carried out in 48-well plates using an ABI StepOnePlus Sequence Detection System (Applied Biosystems, Singapore). All experiments were performed in triplicate.

IMMUNOBLOTTING ANALYSIS

Total cell extracts were harvested in RIPA buffer and centrifuged at 15,000 rpm for 15 min at 4°C. Total protein was quantified by a BCA protein assay (Bio-Rad Laboratories, Hercules, CA). Proteins were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. After blocking with 5% skim milk in PBS-T, membranes were incubated with specific primary antibodies for Tie2, p-Tie2, HO-1, collagen type 2, BMP2, or β-actin (Cell Signaling, Danvers, MA) diluted 1:1,000 in 1% skim milk in PBS-T overnight at 4°C. After washing, the blots were incubated with peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Millipore, Temecula, CA) diluted 1:10,000 in PBS-T at room temperature for 1 h. Signals were detected using the SuperSignal West Dura extended duration substrate (Thermo, CA) according to the manufacturer's instructions. Densitometric analysis was conducted directly from the blotted membrane with a Chemi Imager analyzer system (Alpha Innotech, San Leandro, CA).

TABLE I. Primer Sequences Used in Real-Time RT–PCR

Gene name	Sequences for primers	
Collagen type 2	FOR: GAGTGGAAGAGCGGAGACTACTG REV: CTCCATGTTGCAGAAGACTTTCA	Chondrogenic genes
SOX9	FOR: AGAGCGTTGCTCGGAACTGT REV: TCCTGGACCGAAACTGGTAAA	
BMP2	FOR: TAGTGACTTTTGGCCACGACG REV: GCTTCCGCTGTTTGTGTTTG	Osteogenic genes
RUNX2	FOR: TCCCCGGGAACCAAGAAG REV: CGGTCAGAGAACAAACTAGGTTTAGA	
HO-1	FOR: AGAGTTTCCGCCTCCAACCA REV: CGGGACTGGGCTAGTTCAGG	
β-actin	FOR: ATCGTGGGCCGCCCTAGGCA REV: TGGCCTTAGGGTTCAGAGGGG	

FOR, forward; REV, reverse.

STATISTICAL ANALYSIS

All experiments were repeated at least twice, with each experiment comprising triplicate samples, and qualitatively identical results were confirmed. Statistical analyses were performed using one-way ANOVA followed by a Student-Newman–Keuls test. Data are expressed as the mean ± SEM. Differences with P values < 0.05 were considered statistically significant.

RESULTS

COMP-ANG1 IS NON-TOXIC IN CHONDROCYTES

The toxicity of COMP-Ang1 in chondrocytes at concentrations suitable for subsequent experiments was evaluated by WST assay. The WST assay was used to determine the effect of COMP-Ang1 on the mitochondrial activity of cultured chondrocytes. Figure 2 shows the mitochondrial activity of chondrocytes incubated with different concentrations of COMP-Ang1 for 24 h. The results indicate that there was no significant difference in mitochondrial activity among chondrocytes incubated with different concentrations of COMP-Ang1. The mitochondrial activity of chondrocytes incubated with COMP-Ang1 for 48 or 72 h increased slightly (data not shown). These results suggest that COMP-Ang1 is non-toxic in chondrocytes, and non-toxic concentrations of COMP-Ang1 were used in subsequent experiments.

COMP-ANG1 INDUCES CHONDROCYTE MATURATION

Various growth factors and their downstream signaling pathways regulate chondrocyte maturation [Drissi et al., 2005; Schroeppel

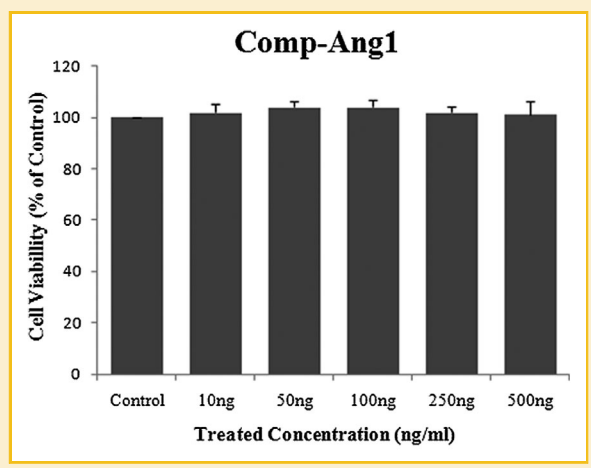

Fig. 2. WST assay to determine COMP-Ang1 toxicity in induced chondrocytes. Various concentrations of COMP-Ang1 (10–500 ng/ml) were added to the culture medium and incubated 24 h. Cell toxicity was assessed by the decrease in WST, expressed as a percentage of untreated control cells grown in a defined medium.

et al., 2011]. These downstream signals often synthesize or activate transcription factors to regulate gene expression. Most potent gene expression in chondrogenesis involves collagen type 2 and SOX9 signaling [Drissi et al., 2005; Tew and Clegg, 2011]. These chondrogenic genes mediate the synthesis of extracellular matrix components to maintain immature chondrocytes. RUNX2 and BMP2 are the most potent genes expressed during osteogenesis and are involved in chondrocyte maturation signaling [Drissi et al., 2005; Wang et al., 2007]. Thus, expression of chondrogenic genes such as collagen type 2 and SOX9 decreases while expression of osteogenic genes such as RUNX2 and BMP2 increases during chondrocyte maturation. We treated chondrocytes with various concentrations of COMP-Ang1 for 12 h. As shown in Figure 3, the COMP-Ang1-treated chondrocytes exhibited a dose-dependent decrease in collagen type 2 and SOX9 expression, while the expression of both RUNX2 and BMP2 increased. These results suggest that COMP-Ang1 induces chondrocyte maturation.

COMP-ANG1 REDUCES HO-1 GENE EXPRESSION AND PROTEIN ABUNDANCE

HO-1 is a multipotent functional factor in various cell types. In chondrocytes, HO-1 upregulates the expression of chondrogenic genes such as collagen type 2 and SOX9 [Guillen et al., 2008], suggesting that HO-1 may regulate chondrocyte metabolism related to chondrocyte maturation. Thus, we hypothesized that COMP-Ang1 decreases HO-1 gene and protein expression in a dose-dependent manner. Total RNA and protein were obtained after incubating chondrocytes with COMP-Ang1 for 12 and 24 h, respectively. As shown in Figure 4A, both the gene and protein expression of HO-1 decreased significantly when chondrocytes were treated with high COMP-Ang1 concentrations. Accordingly, we confirmed that COMP-Ang1 activated Tie2 receptors, which are related to HO-1. As shown in Figure 4B, COMP-Ang1 strongly increased Tie2 phosphorylation and decreased HO-1 protein expression in chondrocytes. However, HO-1 protein expression was remarkably increased by Tie2 siRNA. These results suggest that COMP-Ang1 reduces HO-1 gene and protein expression during chondrocyte maturation through the Ang1/Tie2 signaling pathway.

COMP-ANG1 INCREASED ROS PRODUCTION THROUGH HO-1 REDUCTION

To characterize the events underlying the COMP-Ang1-induced decrease in HO-1 expression, we examined ROS generation after treating chondrocytes with various concentrations of COMP-Ang1. Previous reports have shown that COMP-Ang1 induces dose-dependent ROS production [Kim et al., 2006b]. ROS production is considered to be part of the mechanism of osteogenesis [Marsell and Einhorn, 2011] and induces chondrocyte hypertrophy in endochondral ossification [Morita et al., 2007]. In our study, chondrocytes were exposed to COMP-Ang1 for 6 h, and ROS production was measured as described in the Methods section. As shown in Figure 5A, ROS production in COMP-Ang1-treated chondrocytes increased in a dose-dependent manner. Specifically, chondrocytes treated with 100 ng/ml COMP-Ang1 exhibited a significant increase in ROS production up to

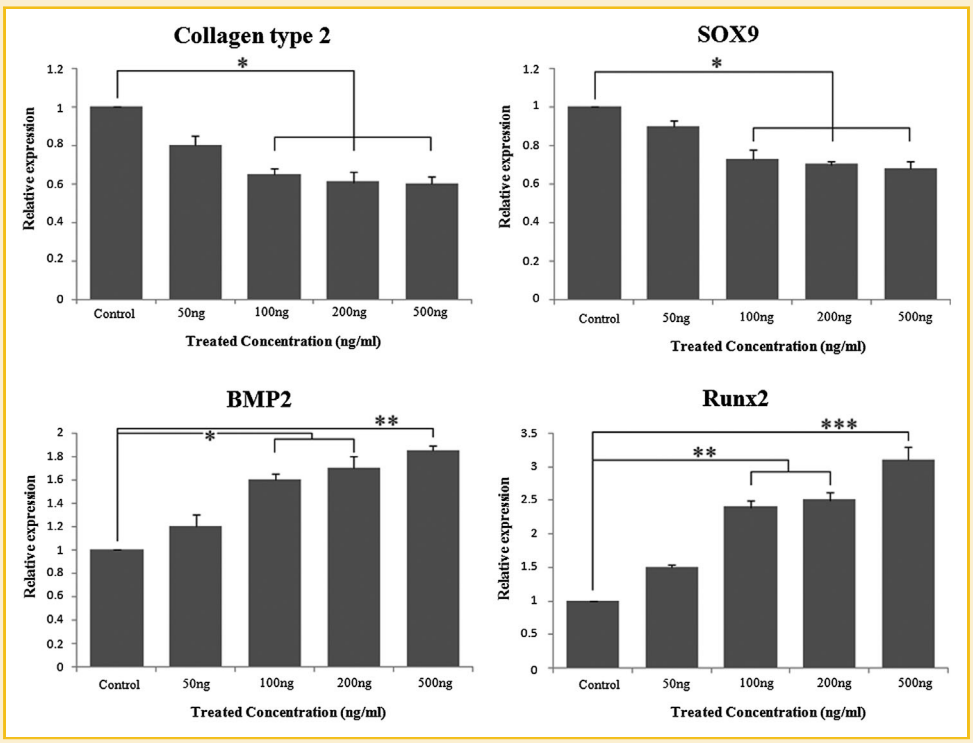

Fig. 3. Real-time RT-PCR analysis comparing chondrogenic and osteogenic gene expression. Real-time RT-PCR analysis of chondrogenic and osteogenic genes was performed as described in the Methods Section. Chondrocytes treated with COMP-Ang1 exhibited significant dose-dependent changes in osteogenic and chondrogenic gene expression (50–500 ng/ml). Each value is the mean ± SEM of triplicate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

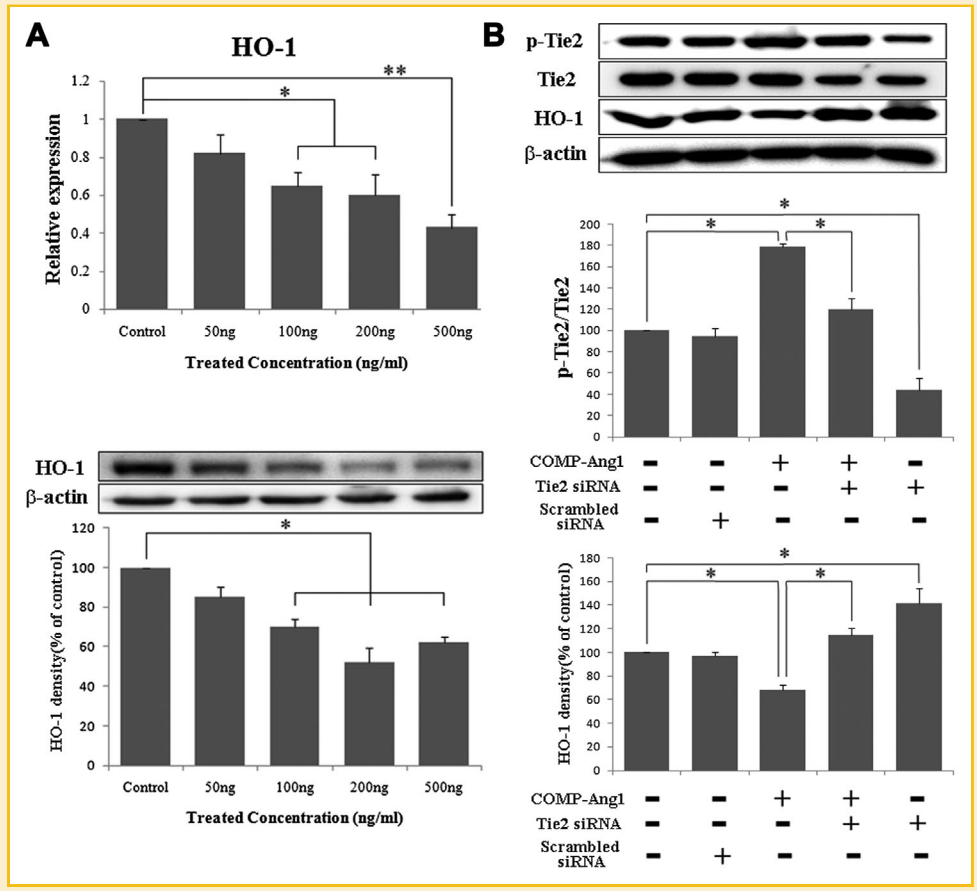

Fig. 4. HO-1 analysis of COMP-Ang1-induced chondrocyte maturation. Chondrocytes were treated with various concentrations of COMP-Ang1 (50–500 ng/ml). Real-time RT-PCR and immunoblotting analysis was performed on COMP-Ang1-treated chondrocytes (A). Immunoblotting was performed to define the relationship between Tie2 and HO-1 protein (B). HO-1 expression decreased significantly in chondrocytes treated with COMP-Ang1, but recovered when treated with Tie2 siRNA. Densitometric results are expressed as the mean ± SEM of triplicate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

1.8-fold higher than the untreated control. We assessed HO-1 expression and ROS production in chondrocytes treated with COMP-Ang1 (100 ng/ml) in the absence or presence of 10 μM SnPP IX, an HO-1 inhibitor, and 10 μM CoPP IX, an HO-1 inducer, after incubating for 6 h. As shown in Figure 5B, COMP-Ang1-treated chondrocytes exhibited HO-1 expression and ROS production similar to that of SnPP IX-treated chondrocytes. Specifically, SnPP IX treatment potentiated COMP-Ang1-induced ROS production up to 1.4-fold higher than chondrocytes treated with only COMP-Ang1. In contrast, CoPP IX slightly abrogated COMP-Ang1-induced ROS

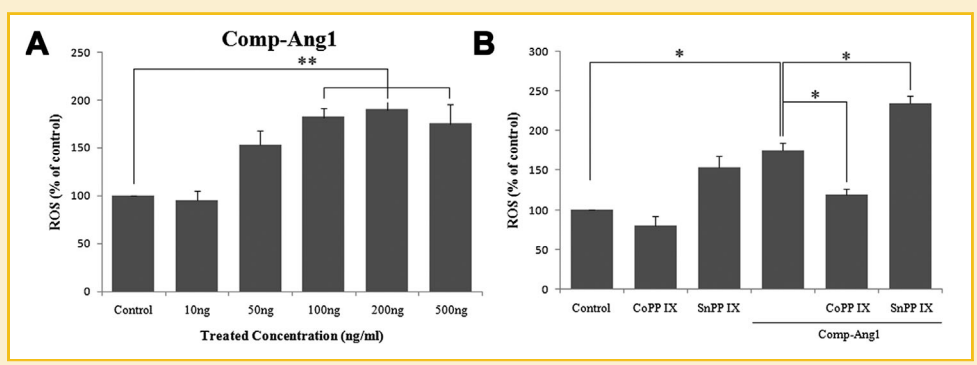

Fig. 5. ROS was induced by COMP-Ang1 with or without CoPP IX and SnPP IX. ROS production was measured in chondrocytes incubated with various concentrations of COMP-Ang1 (50–500 ng/ml) for 6 h (A) and or COMP-Ang1 (100 ng/ml) and 10 μM CoPP IX or 10 μM SnPP IX for 6 h (B). The data are expressed as the mean ± SEM of triplicate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

production. These results suggest that COMP-Ang1 increases ROS production by decreasing HO-1 expression.

COMP-ANG1 ACCELERATES CHONDROCYTE MATURATION BY DECREASING HO-1 EXPRESSION

Based on our findings, we analyzed changes in the gene and protein expression in response to different levels of HO-1. Chondrocytes were treated with 10 μM CoPP IX, 10 μM SnPP IX, and/or COMP-Ang1 (100 ng/ml). Total RNA was obtained after 12 h, and protein extracts were obtained after 24 h. Real-time RT-PCR and immunoblotting for collagen type 2, SOX9, RUNX2, BMP2, and HO-1 expression were performed as described in the Methods Section. As shown in Figure 6, COMP-Ang1 accelerated chondrocyte maturation by decreasing HO-1 expression. Likewise, expression of the collagen type 2 and SOX9

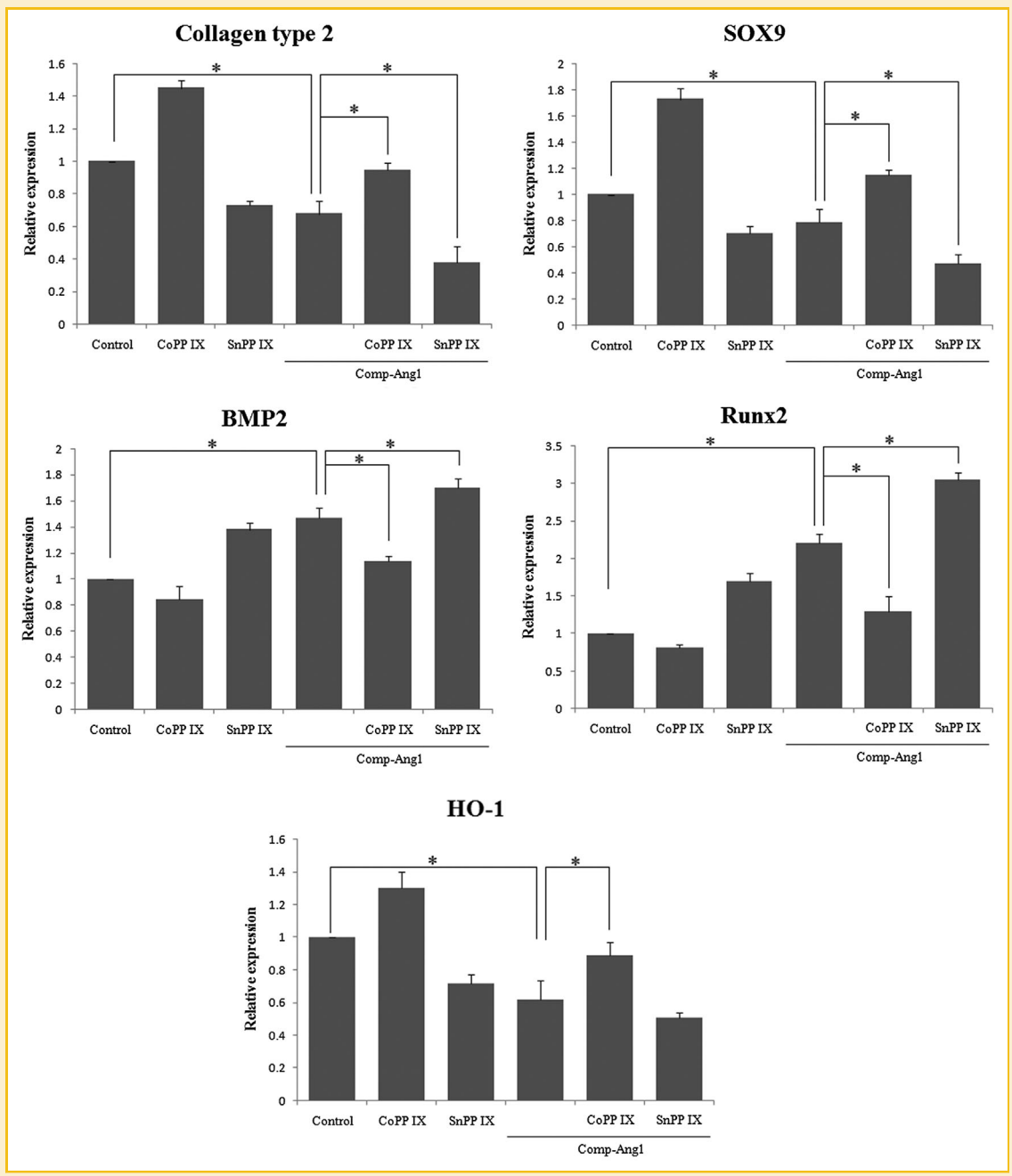

Fig. 6. Real-time RT-PCR analysis of the relationship between HO-1 and COMP-Ang1 treatment during chondrocyte maturation. Total RNA was obtained from chondrocytes treated with COMP-Ang1 (100 ng/ml) and either 10 μM CoPP IX or 10 μM SnPP IX for 12 h. Real-time RT-PCR analysis of osteogenic, chondrogenic, and HO-1 genes was performed as described in the Methods Section. The data revealed an equally significant decrease or increase in gene expression, which was consistent with previous results. Each value is expressed as the mean ± SEM of triplicate experiments $^{*}P < 0.05$.

genes was consistent with HO-1 gene expression. CoPP IX significantly inhibited COMP-Ang1 from reducing collagen type 2 and SOX9 expression. In contrast, SnPP IX further downregulated collagen type 2, SOX9, and HO-1 in addition to the decreased expression caused by COMP-Ang1. However, BMP2 and RUNX2 gene expressions compared with HO-1 gene expression showed the opposite trend. As shown in Figure 7, the expression of BMP2, RUNX2, and HO-1 protein was determined by immunoblotting. Collagen type 2 represents chondrogenic genes, BMP2 represents osteogenic genes, and HO-1 mediates COMP-Ang1-induced chondrocyte maturation. Changes in HO-1 protein expression were consistent with changes in gene expression, suggesting that chondrocyte maturation induced by COMP-Ang1 is associated with reduced HO-1 expression.

DISCUSSION

In vivo and in vitro investigations have provided insight into the pathways that regulate the biologically optimized process of bone formation and have provided a direction for research on the prevention of bone failure. In vitro models have made it possible to investigate bone formation from many perspectives, such as biochemical and signaling pathways, and have therefore been an important tool in understanding osteogenesis [Tare et al., 2010; Marsell and Einhorn, 2011].

Both intramembranous and endochondral ossification are common pathways of bone formation [Bukata, 2011; Marsell and Einhorn, 2011]. Endochondral ossification not only involves angiogenesis, but also chondrocyte maturation and cartilaginous degradation, as cells and extracellular matrix must be removed to allow blood vessel ingrowth [Ai-Aql et al., 2008]. Once this structure is achieved, osteogenesis is mainly regulated by an angiopoietin-dependent pathway [Keramaris et al., 2008]. Angiopoietins, primarily Ang1 and 2, are vascular morphogenetic proteins, and their expression is induced early in the healing cascade, suggesting that they promote initial vascular in growth from existing vessels in the periosteum [Lehmann et al., 2005]. Ang1 promotes both vasculogenesis, the aggregation and proliferation of endothelial mesenchymal stem cells into a vascular plexus, and angiogenesis, the growth of new vessels from existing structures [Asahara et al., 1998]. Hence, Ang1 plays a crucial role in neoangiogenesis and revascularization during osteogenesis [Fang et al., 2005]. The importance of Ang1 in these processes is supported by the observation that adding excessive Ang1 promotes bone formation, whereas blocking Tie2, the angiopoietin receptor, inhibits vascular ingrowth and delays or disrupts regeneration. In addition, osteoblast-mediated overexpression of Ang1 reportedly increases ALP activity and bone mass in vivo, suggesting that Ang1 is also coupled to bone formation [Suzuki et al., 2007]. Furthermore, increased Ang1 expression during angiogenesis-induced chondrocyte maturation involving endochondral ossification has been demonstrated [Fang et al., 2005; Jeong et al., 2010; Park et al., 2010]. Although using Ang1 to enhance osteogenesis has many advantages, the coiled-coil domain and N-terminal superclustering domain responsible for the modular and multimeric structure of Ang1 results in protein aggregation and insolubility, which makes producing a recombinant protein difficult and limits its clinical use. To overcome these problems, recombinant COMP-Ang1 protein, a chimeric form of Ang1 containing a minimal coiled-coil domain to allow oligomerization, was synthesized. COMP-Ang1 has many potential advantages over native Ang1, including the efficiency of generation, potency, and Tie2 activation. In a previous study, the efficacy of COMP-Ang1 for inducing osteogenesis was evaluated by injecting COMP-Ang1 into the distraction gap in a rat model. Gross radiological and histological evidence indicated that COMP-Ang1 accelerated the formation of new bone as a result of endochondral ossification [Park et al., 2010]. Thus, we provided

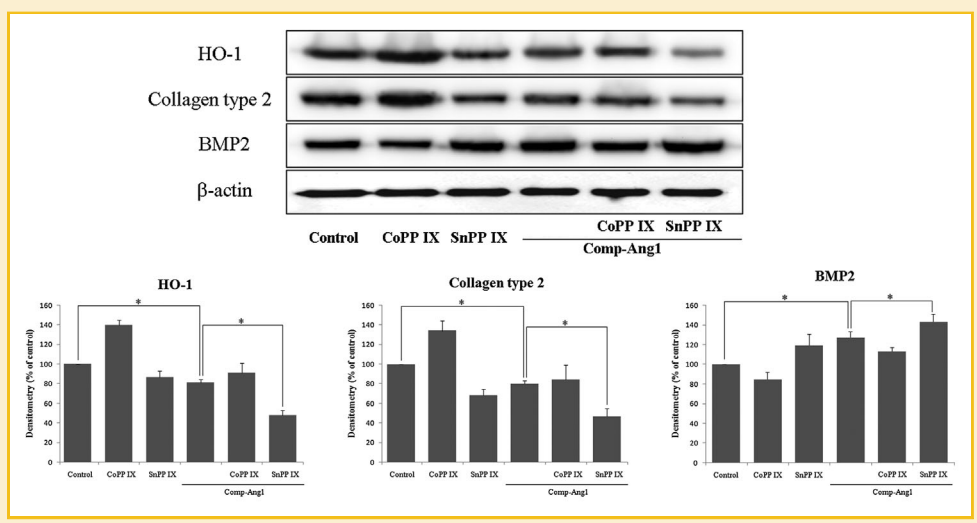

Fig. 7. Immunoblotting analysis of HO-1 in COMP-Ang1-treated chondrocytes. Protein extracts from chondrocytes treated with COMP-Ang1 (100 ng/ml) and 10 μM CoPP IX or 10 μM SnPP IX for 24 h. Immunoblotting for BMP2, collagen type 2, and HO-1 protein was performed as described in the Methods section. These results revealed equally significant decreases or increases in protein expression consistent with the gene expression data. Each value is expressed as the mean ± SEM of triplicate experiments. $^{*}P < 0.05$.

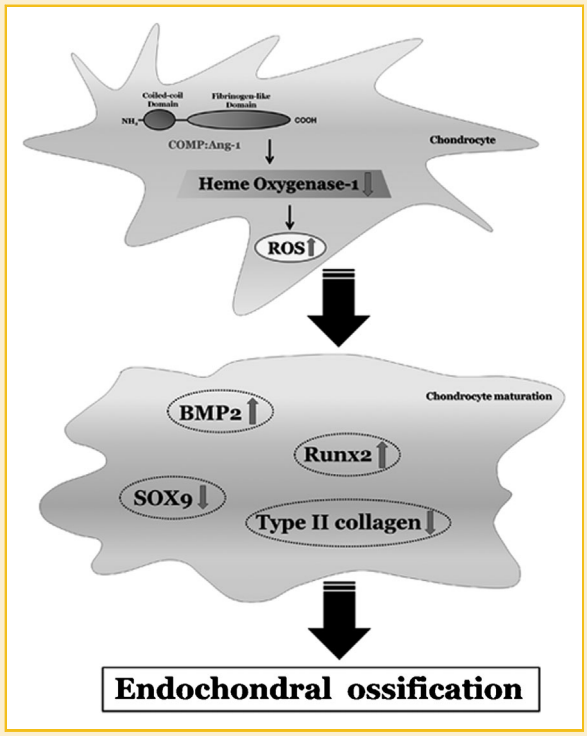

Fig. 8. Schematic diagram of COMP-Ang1-induced chondrocyte maturation. COMP-Ang1 reduces HO-1 expression, which increases ROS production. According to these results, COMP-Ang1 accelerated chondrocyte maturation by increasing osteogenic gene expression and decreasing chondrogenic gene expression.

evidence that COMP-Ang1-treated chondrocyte maturation is involved in endochondral ossification in vitro. Specifically, COMP-Ang1 enhanced osteogenic gene expression and decreased HO-1 expression, facilitating chondrocyte maturation.

Several lines of evidence have demonstrated that the inducible isoforms of HO-1 participate in the maintenance of cellular homeostasis and plays an important protective role by reducing ROS production. Due to its regulatory pattern, the induction of HO-1 has generally been considered an adaptive response against cellular stimulation [Ryter and Choi, 2010]. However, a recent study of HO-1 highlighted the effect of CoPP IX, an HO-1 inducer, in increasing collagen type 2, a chondrogenic gene that maintains the immature chondrocyte phenotype [Guillen et al., 2008]. In our study, chondrocytes treated with COMP-Ang1 exhibited dose-dependent decreases in HO-1 expression and increased Tie2 phosphorylation, decreasing HO-1 expression (Fig. 4). ROS production increased in a dose-dependent manner with decreased HO-1 expression in COMP-Ang1-treated chondrocytes (Fig. 5). Previous studies have already demonstrated that COMP-Ang1 stimulates ROS production [Kim et al., 2006b] and that ROS production induces osteogenesis [Morita et al., 2007]. To clarify the relationship between HO-1 and COMP-Ang1 in chondrocytes, we used an HO-1 inducer, CoPP IX, and an HO-1 inhibitor, SnPP-IX. We confirmed that COMP-Ang1 accelerated chondrocyte maturation by abrogating HO-1 expression. HO-1 inhibition induced BMP2 and RUNX2 and reduced collagen type 2 and SOX9 expression in chondrocytes (Figs. 6 and 7). Taken together, these results suggest that a reduction in HO-1 expression induced by COMP-Ang1 is essential for chondrocyte maturation.

In summary, the present study showed that COMP-Ang1 synergistically accelerated chondrocyte maturation, which is involved in endochondral ossification, by reducing HO-1 expression (Fig. 8). Thus, the results of our study suggest a potential therapeutic relationship between COMP-Ang1 and HO-1 in healing bone fractures.

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