HIF-1α as a Regulator of BMP2-Induced Chondrogenic Differentiation, Osteogenic Differentiation, and Endochondral Ossification in Stem Cells

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Key Words
Hypoxia inducible factor-1α • Bone morphogenetic protein 2 • Stem cell • Chondrogenic differentiation • Osteogenic differentiation • Cartilage tissue engineering

Abstract

Background/Aims: Joint cartilage defects are difficult to treat due to the limited self-repair capacities of cartilage. Cartilage tissue engineering based on stem cells and gene enhancement is a potential alternative for cartilage repair. Bone morphogenetic protein 2 (BMP2) has been shown to induce chondrogenic differentiation in mesenchymal stem cells (MSCs); however, maintaining the phenotypes of MSCs during cartilage repair since differentiation occurs along the endochondral ossification pathway. In this study, hypoxia inducible factor, or (HIF)-1α, was determined to be a regulator of BMP2-induced chondrogenic differentiation, osteogenic differentiation, and endochondral bone formation. Methods: BMP2 was used to induce chondrogenic and osteogenic differentiation in stem cells and fetal limb development. After HIF-1α was added to the inducing system, any changes in the differentiation markers were assessed. Results: HIF-1α was found to potentiate BMP2-induced Sox9 and the expression of chondrogenesis by downstream markers, and inhibit Runx2 and the expression of osteogenesis by downstream markers in vitro. In subcutaneous stem cell implantation studies, HIF-1α was shown to potentiate BMP2-induced cartilage formation and inhibit endochondral ossification during ectopic bone/cartilage formation. In the fetal limb culture, HIF-1α and BMP2 synergistically promoted the expansion of the proliferating chondrocyte zone and inhibited chondrocyte hypertrophy and endochondral ossification. Conclusion: The results of this study indicated that, when combined with BMP2, HIF-1α induced MSC differentiation could become a new method of maintaining cartilage phenotypes during cartilage tissue engineering.

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Introduction

Joint cartilage defects resulting from degenerative disorders or traumatic injuries are difficult to treat due to the cartilage’s limited capacity for self repair [1]. Numerous surgical methods have been applied in an effort to repair cartilage, reduce joint pain, and improve joint function, primarily marrow stimulating techniques and transplantation techniques [2]. However, marrow stimulating techniques, such as abrasion arthroplasty, pridie drilling, and microfracture, have failed to generate satisfactory clinical results [2, 3]. In addition, the application of transplantation techniques, such as osteochondral autologous transplantation and autologous chondrocyte implantation, are limited by insufficient amounts of healthy non-weight-bearing joint cartilage, donor site morbidity, loss of chondrocytic phenotypes during expansion, and immunological reactions [2, 3]. As a result, the use of stem cells and gene enhancement to engineer cartilage has become a viable alternative to other cartilage repair methods [1, 2, 4].

Isolated from bone marrow [5], trabecular bone chips [6], adipose tissue [7], periosteum [8], perichondrium [9] and other tissues [10], mesenchymal stem cells (MSCs) have been proven to be capable of expanding through multiple passages without losing their ability to undergo chondrogenic, osteogenic, and adipogenic differentiation [11-14]. Because of this, MSCs are considered ideal seed cells for cartilage tissue engineering. However, the successful generation of stable hyaline cartilage tissue using MSCs requires the efficient delivery of the factors stimulating chondrogenesis [1, 15]. Many studies have reported that certain growth factors, such as transforming growth factor (TGF)-βs [16, 17], bone morphogenetic proteins (BMPs) [18, 19], fibroblast growth factors (FGFs) [20], and insulin-like growth factor (IGF)-1 [21, 22], are capable of directing MSCs to chondrocyte phenotypes. However, these growth factors have not been extensively used due to their limited abilities to synthesize specific cartilage matrix components. Hence, the optimization of growth factors and the regulation of their contributions to chondrogenesis is crucial.

Bone morphogenetic protein 2 (BMP2), which belongs to the TGF-β super-family, plays an essential role in the regulation of chondrocyte proliferation and maturation during endochondral bone development in vivo [23]. This protein has also been proven to induce chondrogenic differentiation in various types of stem cells, such as human mesenchymal stem cells (hMSCs) [24], adipose-derived stem cells (ADSCs) [22], and mouse embryonic fibroblasts (MEFs) [25], in vitro. BMP2 has also been proven to be more capable of inducing MSC chondrogenic differentiation than other growth factors, such as TGF-βs and IGF-1 [26]. However, BMP2 also induces MSC osteogenic differentiation [24] and stimulates endochondral ossification [23]. Thus, potentiating BMP2-induced MSC chondrogenic differentiation while inhibiting BMP2-induced MSC osteogenic differentiation and endochondral ossification could be vital to maintaining hyaline-like cartilage phenotypes during cartilage tissue engineering. Sox9 is known to be a critical factor during chondrogenic differentiation [25], but Runx2 is a critical factor during osteogenic differentiation and endochondral ossification [27] that stimulates hypertrophic chondrocyte maturation [28]. Both of these factors can be regulated by BMP2 during the osteochondrogenic differentiation of stem cells in vivo and vitro [25, 27]. Therefore, potentiating Sox9 expression and inhibiting Runx2 expression via regulator optimization could promote BMP2-induced MSC chondrogenic differentiation, inhibit endochondral ossification, and maintain cartilage phenotypes.

Hypoxia inductive factor, or (HIF)-1α, which is a master regulator of hypoxia, plays an important role during the osteochondrogenic differentiation of stem cells [29, 30]. HIF-1α has been proven to bind to Sox9 promoters, activate Sox9 expression, and enhance Sox9-mediated transcriptional activity during chondrogenesis [30-32]. HIF-1α has also been shown to inhibit MSC osteogenesis by directly downregulating Runx2 via TWIST [33]. Some studies have concluded that the stabilization of HIF-1α in normoxic conditions could be a new method of stabilizing cartilage phenotypes [34]. Thus, HIF-1α could adequately regulate BMP2-induced MSCs chondrogenic differentiation, osteogenic differentiation,
and endochondral ossification, cartilage phenotype maintenance during cartilage tissue engineering.

In this study, the use of HIF-1α to potentiate BMP2-induced MSC chondrogenic differentiation, inhibit osteogenic differentiation and endochondral ossification, and maintain cartilage phenotypes was investigated. HIF-1α was determined to be capable of potentiating BMP2-induced Sox9 expression and the expression of chondrogenesis in downstream markers, while inhibiting Runx2 and the expression of osteogenesis in downstream markers in vitro. In the subcutaneous stem cell implantation studies, HIF-1α was proven to potentiate BMP2-induced cartilage formation and inhibit endochondral ossification during ectopic bone/cartilage formation. In the fetal limb culture, HIF-1α and BMP2 were determined to synergistically promote the expansion of the proliferating chondrocyte zone and inhibit chondrocyte hypertrophy and ossification. The results of this study indicated that BMP2, when combined with HIF-1α, induced MSC differentiation and could become a new method of maintaining chondrocyte phenotypes during cartilage tissue engineering.

**Materials and Methods**

**HEK-293 cells and the C3H10T1/2 cell line culture**

The HEK-293 cells and mesenchymal stem cell line (C3H10T1/2) used in this study were purchased from the American Type Culture Collection (ATCC; USA). They were maintained in Dulbecco modified Eagle medium (DMEM) (Hyclone, USA) supplemented with 10% fetal bovine serum (Hyclone, USA), 100 U penicillin, and 100 mg streptomycin (Beyotime, China). They were incubated at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air. The cells were passaged with trypsin (Hyclone, USA) when they were approximately 85% confluent.

**Recombinant Adenoviruses Expressing GFP, BMP2, and Sox9**

Recombinant adenoviruses were generated using AdEasy technology [35]. The human BMP2 and human HIF-1α coding regions were polymerase-chain-reaction (PCR)-amplified, cloned into an adenoviral shuttle vector, and subsequently used to generate recombinant adenoviruses in the HEK-293 cells. The primers used to amplify the coding regions included HIF-1α F: 5’-AGGGGATCCACCATGGAGGGCGCCGGCG CGCGAACGAC-3’, R:5’-TGCTCTAGATTAGTTAACTTGATCCAAAGCTCTGAG-3’; BMP2 F: 5’-CCCAAGCTTACCA CCCATGCTGGCCGGACCCCGCTCTTC-3’, R: 5’-CGGGGATCCCTAGCGACACCCACAACCCTCACAC-3’; BamH I and Xba I enzymes were used to clone the HIF-1α coding regions into the shuttle vector. BamH I and Hind3 enzymes were used to clone the BMP2 coding regions into the shuttle vector. The resulting adenoviruses were denoted AdBMP2, AdHIF-1α, and AdGFP. AdBMP2 also expressed green fluorescent protein (GFP), whereas AdHIF-1α expressed red fluorescent protein (RFP) markers, which were used to monitor the infection efficiency via fluorescence microscopy (Nikon TE200-U, Japan). AdGFP, which only expressed GFP, was used as the control.

**Micromass chondrogenesis cultures**

Micromass cultures created with C3H10T1/2 cells were used to effectively study in vitro chondrogenesis since these cells do not spontaneously differentiate under normal culture conditions [36], necessitating the addition of exogenous factors, such as BMP2 and HIF-1α, into the culture medium [37]. This made it easier to study the effects of these factors on the C3H10T1/2 cell differentiation potential. Twenty-four hours after AdBMP2, AdHIF-1α, AdBMP2+AdHIF-1α, and AdGFP were injected, the micromass cultures were prepared as previously described [36, 37]. The cultures were grown at a density of 1.5 × 10⁷ cells·mL⁻¹ in DMEM supplemented with 10% FBS; then, 40-µL cell suspension droplets were inoculated into the centers of 12-well plates (Corning, USA). Then, after the cells were allowed to combine for 1.5 hours at 37°C under 5% CO₂, the plates were flooded with the culture medium, which was changed every other day. The day of inoculation was considered to be culture day zero. The micromass cultures were maintained for up to nine days.
**Alcian blue staining**

The sulfated glycosaminoglycans (GAGs) produced by the micromass cultures were investigated using Alcian blue staining after seven and nine days. The micromass cultures were rinsed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 15 minutes. They were subsequently stained with Alcian blue solution (Sigma-Aldrich, Germany) at room temperature as previously described [37] and then photographed.

**Alkaline phosphatase (ALP) activity assays**

C3H10T1/2 cells were seeded in 24-well plates and infected with AdBMP2, AdHIF-1α, AdBMP2+AdHIF-1α, and AdGFP. Seven and nine days after the infection, the samples were fixed with a fixative solution containing citrate and acetone, for 30 seconds at room temperature. Next, the cell samples were incubated with Alkaline Dye Mixture (Sigma-Aldrich, Germany) at room temperature for 10–60 minutes, and the resulting staining were monitored under a microscope (Nikon TE200-U) every 5 minutes. Photographs were collected. Each of the chemiluminescence assay conditions were replicated three times. The tests were replicated in at least three independent experiments.

**Calcium deposition**

C3H10T1/2 cells were seeded in 24-well plates and infected with AdBMP2, AdHIF-1α, AdBMP2+AdHIF-1α, and AdGFP. The infected cells were cultured in a mineralization medium containing ascorbic acid (50 μg/mL) and β-glycerophosphate (10 mM). Seven and nine days after infection, the mineralized matrix was stained for calcium precipitation using Alizarin red S staining as previously described [36, 38]. The cells were fixed with 0.05% (v/v) glutaraldehyde at room temperature for 10 minutes. After being washed with distilled water, the fixed cells were incubated with 0.4% Alizarin red S (Sigma-Aldrich, Germany) for 5 minutes then extensively washed with distilled water. The calcium mineral deposit stainings were photographed under a microscope (Nikon TE200-U).

**Cell for RNA isolation and protein isolation**

C3H10T1/2 cells were seeded at a density of 1 × 10^5 cells/well in six-well plates (Corning) containing DMEM supplemented with 10% FBS, and then infected with AdBMP2, AdHIF-1α, AdBMP2+AdHIF-1α, and AdGFP. The culture medium was changed on every second day. They were incubated at 37°C in a humidified atmosphere containing 5% CO2 and 95% air.

**RNA isolation and semiquantitative reverse transcription-PCR**

Total RNA was extracted with Trizol reagent (Invitrogen, USA) at different times. An iScript cDNA synthesis kit (Bio-Rad, USA) was used to generate cDNA templates from the total RNA through reverse transcription (RT). The first-strand cDNA products were further diluted and used as PCR templates. The PCR primers (Table 1) were designed using Primer 3.0 (ABI Coperation, USA) to amplify the genes of interest (approximately 150–180 bp). Semiquantitative RT-PCR was conducted as described previously [39]. The cDNA was amplified in a 25-μL PCR mix. The following touchdown cycling program was used: 94°C for 2 min for 1 cycle; 92°C for 20 s, 68°C for 30 s, and 72°C for 12 cycles decreasing 1°C per cycle; and then 92°C for 20 s, 57°C for 30 s, and 72°C for 20 s for 20–25 cycles, depending on the abundance of a given gene. The PCR products were resolved on 2% agarose gel. All of the samples were normalized with the GAPDH expression level.

### Table 1. Oligonucleotide primers utilized for RT-PCR amplification

| Target   | Forward primer sequence | Reverse primer sequence | Base pairs |
|----------|-------------------------|-------------------------|------------|
| GAPDH    | 5'-CTCAGTGGAGACGCTTCT-3' | 5'-TTGCTACCCGAAATGACCT-3' | 123bp |
| HIF-1α   | 5'-TGATCCTCTGTGAGCAAAC-3' | 5'-CTTGGCTCAAATGACCT-3' | 152bp |
| BMP2     | 5'-GGTGGAGCTCACGAGAAAT-3' | 5'-CATATGAGCAAGTTCT-3' | 122bp |
| Col2A1   | 5'-AACACGATCCATGAGGAC-3' | 5'-TCTGAGGACATTCCTCT-3' | 159bp |
| Aggrecan | 5'-GGGCTGCTGCTGCTGCTGCT-3' | 5'-TCTGGAGCAGTACG-3' | 188bp |
| Col1A1   | 5'-GGGAGCTGTTTTCATGACG-3' | 5'-AGCCTTGTGTTCTGACG-3' | 150bp |
| ALP      | 5'-GCTCCCGAGATGCTGAG-3' | 5'-AGGCTGCTGTTGACG-3' | 121bp |
Real-time PCR

Real-time PCR was conducted as described previously [40]. The cDNA was amplified in a 10-μL PCR mix containing 5 μL of SYBR Green Super Mixture (Bio-Rad). A real-time PCR was conducted with a CFX-Connect Real-Time PCR system (Bio-Rad) for 3 minutes at 95°C, followed by 40 cycles at 95°C for 10 seconds and 58°C for 5 seconds. The efficiency and specificity values of each primer set were confirmed by comparing the standard curves of the threshold cycle (C_t) values to the RNA serial dilutions and melting profile evaluations. The C_t values were normalized using GAPDH in order to manage any cDNA quantification differences. The results were reported as relative expression levels.

Western blotting analysis

Cells were collected using a Lysis Buffer (Beyotime, China). The cleared total cell lysate was denatured via boiling and resolved via 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. After electrophoretic separation, the proteins were transferred to a polyvinylidene fluoride membrane (Bio-Rad). The membrane was blocked with 5% skim milk for 1 hour at room temperature, and probed overnight with anti-Sox9, anti-Runx2, anti-COL2A1, anti-OPN, anti-OCN, and anti-β-actin (Santa Cruz Biotechnology, USA; 1:500) at 4°C. After five washes, the blots were incubated with a goat anti-rabbit or rabbit anti-mouse peroxidase-conjugated secondary antibody (Abcam, USA; 1:1000) for 2 hours at room temperature. The blots were displayed with Immobilon Western Chemiluminescent HRP Substrate (Millpore, USA). The results of the relative protein expression levels were compared to that of β-actin using Quantity One software (Bio-Rad, Quantity One 4.6.2).

Subcutaneous stem cell implantation

C3H10T1/2 cells were infected with AdBMP2, AdHIF-1α, AdBMP2+AdHIF-1α, and AdGFP. Twenty-four hours after infection, the cells were harvested and resuspended in PBS for subcutaneous injection (5 × 10⁶/injection) into the flanks of athymic nude (nu/nu) mice (groups of four 4- to 6-week-old males, Beijing HFK Bioscience Corporation, China). All of the experiments involving animals were performed according to institutional animal guidelines (Ethics Committee of Chongqing Medical University). Five weeks after implantation, the animals were euthanized, and the bone masses of the implantation sites were retrieved for histological evaluation and other staining procedures.

Hematoxylin and eosin, Alcian blue, safranin O-fast green, and Masson trichrome staining

The retrieved tissues were fixed in 4% paraformaldehyde, decalcified, and embedded in paraffin. Serial sections of the embedded specimens were stained with hematoxylin and eosin, Alcian blue, safranin O-fast green, and Masson trichrome staining according to the previously described protocols [38].

Mouse Fetal Limb Explant Cultures

The forelimbs of mouse embryos (E18.5) were dissected under sterile conditions and incubated in DMEM (Hyclone, China), which contained 0.5% FBS (Gibco, Australia), 50 mg/ml ascorbic acid, 1 mM β-glycerophosphate, 100 U/ml penicillin, and 100 mg/ml streptomycin, at 37°C in humidified air with 5% CO₂ for as many as fourteen days [38]. The limbs were directly infected with AdBMP2, AdHIF-1α, AdBMP2+AdHIF-1α, and AdGFP 12 hours after dissection. Approximately 50% of the culture medium was replaced every two days. On day twelve, 100 mM calcein (Sigma) was added to the medium in order to observe any new bone formation. The cultured forelimbs were observed under a microscope at different times in order to confirm the tissue cell survival and fluorescent marker expression. Then, the forelimb developments were analyzed.

Statistical analysis

Each of the quantitative assay conditions were replicated three times, and all of the results were validated with a minimum of three independent experiments. The data was expressed in terms of mean±standard deviation (SD). Any statistically significant differences among the samples were assessed using a one-way variance analysis (SPSS cooperation, SPSS 17.0), in which a p-value less than 0.05 (p < 0.05) was considered statistically significant.
Results

The C3H10T1/2 cells infected with AdBMP2, AdHIF-1α, or AdGFP exogenously expressed BMP2 and HIF-1α

In order to determine the effects of HIF-1α on BMP2-induced chondrogenic and osteogenic differentiation, recombinant adenoviruses that expressed mouse BMP2 and human HIF-1α during normoxia were generated. The AdHIF-1α, AdBMP2, and AdGFP adenoviruses effectively infected the C3H10T1/2 cells, as was indicated by the visualization of green and red fluorescence. Seventy percent of the cells were positive for GFP and 50% of the cells were positive for RFP 24 hours after infection (Fig. 1A). The semiquantitative RT-PCR analysis revealed HIF-1α and BMP2 expression in the C3H10T1/2 cells infected with AdHIF-1α and AdBMP2, respectively, but not in those infected with AdGFP.

The data indicated that the AdBMP2- and AdHIF-1α-infected cells effectively expressed BMP2 and HIF-1α, respectively, during normoxia. Thus, these cells were suitable for further experimental use.

HIF-1α potentiated BMP2-induced chondrogenic differentiation in the micromass cultures in vitro

The effects of HIF-1α on BMP2-induced chondrogenic differentiation in the C3H10T1/2 cells were investigated using the micromass cultures. The C3H10T1/2 cells were infected with AdBMP2, AdHIF-1α, AdBMP2+AdHIF-1α, and AdGFP and developed in micromass cultures. The resulting micromasses, which were established 24 hours later, were examined for green and red fluorescence in order to monitor the infection efficiencies of the adenoviruses (Fig. 2A). Using the western blotting method, chondrogenic differentiation was evaluated by determining whether Sox9 protein, a critical factor for chondrogenesis, was expressed. Compared to the BMP2 group, the HIF-1α component of the BMP2+HIF-1α group significantly enhanced BMP2-induced Sox9 protein expression on days one and three (P < 0.05) (Figs. 2B and 2C).

The expression of the Sox9 downstream response genes, type II collagen (COL2A1) and aggrecan, was also evaluated at the mRNA level using real-time PCR after five, seven, and nine
days. HIF-1α significantly increased the expression of both of these chondrogenic markers ($P < 0.05$) (Figs. 3A and 3B). In addition, an examination of the COL2A1 protein expression yielded the same results as the mRNA expression examination ($P < 0.05$) (Figs. 3C and 3D). In order to investigate the production of cartilaginous matrix components, the cultures were stained with Alcian blue. The results indicated that HIF-1α enhanced the BMP2-induced secretion of sulfated GAGs in the cartilaginous matrix after seven and nine days (Fig. 3E).

These results suggested that HIF-1α could potentiate BMP2-induced chondrogenic differentiation in vitro.

**HIF-1α inhibited BMP2-induced osteogenic differentiation in the plate culture in vitro**

In addition to its role in chondrogenesis, BMP2 is involved in osteogenesis since it activates Runx2. Thus, the effects of HIF-1α on BMP2-induced osteogenic differentiation were investigated. Western blot assays conducted one and three days after culturing indicated that HIF-1α obviously inhibited the BMP2-induced expression of Runx2 during osteogenesis ($P < 0.05$) (Figs. 4A and 4B).

ALP and type I collagen (COL1A1) are the downstream response genes of Runx2. Therefore, the expression of ALP and COL1A1 mRNA was evaluated using real-time PCR on days seven and nine. HIF-1α inhibited BMP2-induced ALP and COL1A1 mRNA expression ($P$
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< 0.05) (Figs. 4C and 4D). In order to further explore the inhibitory effects of HIF-1α on BMP2-induced osteogenic differentiation, ALP activity assays were conducted at varying times. The results of the staining and relative activity assays indicated that HIF-1α also significantly inhibited BMP2-induced ALP activity on days seven and nine (P < 0.05) (Figs. 4E and 4F).

The expression of the late osteogenic markers, osteocalcin (OCN) and osteopontin (OPN), were also examined using western blot assays. The OCN and OPN protein expression was reduced by HIF-1α; moreover, the relative expression of OCN and OPN was significantly lower in the AdBMP2+AdHIF-1α group than in the AdBMP2 group on days seven and nine (P < 0.05) (Figs. 5A and 5B). In order to conduct the matrix mineralization assays, C3H10T1/2 cells were infected with the indicated adenoviruses and cultured in a mineralization medium. Alizarin red S staining was completed seven and nine days after infection. The staining indicated that HIF-1α inhibited BMP2-induced late-stage osteogenic differentiation (Fig. 5D).

These results demonstrated that HIF-1α could significantly inhibit BMP2-induced early- and late-stage osteogenic differentiation in vitro.
HIF-1α inhibited BMP2-induced osteogenic differentiation and endochondral ossification and maintained the chondrocyte phenotypes during the stem cell implantations in vivo

The above assays established that HIF-1α played two different roles during BMP2-induced chondrogenic differentiation and osteogenic differentiation in vitro. BMP2 was involved in the regulation of chondrocyte development and in some activities during endochondral ossification [23]. Therefore, in this study, we aimed to demonstrate the role of HIF-1α during BMP2-induced osteochondrogenic differentiation in vivo. The C3H10T1/2 cells were determined to be effectively infected with AdBMP2, AdHIF-1α, AdBMP2+AdHIF-1α, and AdGFP, as previously indicated. The infected cells were collected and injected subcutaneously into the athymic nude mice.

After 5 weeks, the animals were euthanized, and the bony masses were retrieved (Fig. 6A). The C3H10T1/2 cells infected with either AdHIF-1α or AdGFP alone did not yield any detectable masses (data not shown). The bony mass volumes of the AdBMP2 and AdBMP2+AdHIF-1α groups did not significantly differ (P > 0.05) (Fig. 6B). Based on a
histological examination conducted with hematoxylin and eosin, Alcian blue, Safranin O-fast green, and Masson trichrome staining, the cells stimulated with both BMP2 and HIF-1α were formed more cartilage with minimal bone matrices and trabeculae, but the BMP2-transduced cells formed less cartilage and more mature bone matrices and thicker trabeculae, with some hypertrophic chondrocytes (Figs. 6C and 6D). The AdBMP2+AdHIF-1α groups had higher trabecular area percentages than the AdBMP2 groups ($P < 0.05$) (Fig. 6E).

From the results obtained in vivo, HIF-1α was found to be capable of enhancing BMP2-induced chondrogenesis while inhibiting the formation of bone matrices, trabeculae, and endochondral ossification; thus, it maintained the chondrocyte phenotypes during osteochondrogenic differentiation in vivo.
HIF-1α promoted the expansion of the proliferating chondrocyte zone and inhibited BMP2-induced chondrocyte hypertrophy and endochondral ossification in the fetal limb explant cultures.

In order to precisely observe the effects of HIF-1α on the BMP2-induced progression of chondrogenesis and endochondral ossification, fetal limb cultures were conducted. The skinned fetal limbs were isolated from the E18.5 perinatal mouse embryos and cultured in an organ culture medium in the presence of AdGFP, AdBMP2, and/or AdHIF-1α for fourteen...
days. The limbs were successfully infected with indicated recombinant adenoviruses on day two (Figs. 7A-C). The ossification of new bone was traced by the fluorescent dye, calcein, on day fourteen (Figs. 7D-F).

Based on the histological examination, BMP2 induced chondrocyte proliferation, hypertrophy, and endochondral ossification. When the limbs were co-infected with AdBMP2

Fig. 7. Mouse E18.5 forelimbs (n = four mice in each group) were cultured in an organ culture medium, and their transduction efficiencies and the ossification of new bone dyed with calcein were visualized under a bright fluorescent field (40X). A: The forelimbs in the bright field on day 2. B: The forelimbs in the green fluorescent field on day 2. C: The forelimbs in the red fluorescent field on day 2. D: The forelimbs dyed with calcein in the bright field on day 14. E: The forelimbs dyed with calcein in the green fluorescent field on day 14. F: The forelimbs dyed with calcein in the red fluorescent field on day 14.

Fig. 8. The effects of HIF-1α on BMP2-induced skeletal development as determined by the fetal limb culture assay. A: Histological analysis of the cultured forelimbs. The forelimbs were subjected to H&E staining. Representative images are shown (100X). B: The average lengths of the hypertrophic zones, prehypertrophic zones, and proliferating zones were determined using Image J software. C: The green fluorescence (calcein staining) signal in the ulna of a cultured limb (40X). D: A quantitative analysis of the bone-forming activity (based on calcein incorporation) was conducted using Image J. Hyp = hypertrophic chondrocyte zone, Pre = prehypertrophic chondrocyte zones, Pro = proliferating chondrocyte zone. The data us denoted in terms of mean ± SD. *: P < 0.05, BMP2 vs. BMP2+HIF-1α.
and AdHIF-1α, the proliferating chondrocyte zones expanded while the hypertrophic chondrocyte zone did not (Fig. 8A). The quantitative analysis of the histologic data also indicated that the hypertrophic chondrocyte zone treated with BMP2 and HIF-1α was shorter than that treated with BMP2 alone \( (P < 0.05) \) (Fig. 8B). The limb treated with both BMP2 and HIF-1α exhibited less bone formation than that treated with BMP2 alone, as shown by the calcine uptake \( (P < 0.05) \) (Figs. 8C-D).

These results suggested that HIF-1α promoted the expansion of the proliferating chondrocyte zone and inhibited BMP2-induced chondrocyte hypertrophy and endochondral ossification in the fetal limb.

**Discussion**

Stem-cell-based, gene-enhanced cartilage tissue engineering could be a feasible alternative to cartilage repair [1, 2]. BMP2 has been shown to be capable of inducing chondrogenic differentiation in MSCs [22, 24-26]. However, maintaining the phenotypes of MSCs during cartilage repair is very difficult due to the differentiation that occurs along the endochondral ossification pathway during that process [41]. In this study, HIF-1α was selected as a regulator of BMP2-induced chondrogenic differentiation, osteogenic differentiation, and endochondral bone formation. HIF-1α was found to potentiate BMP2-induced MSCs chondrogenic differentiation and inhibit osteogenic differentiation and endochondral ossification. This regulation could allow for the maintenance of hyaline-like cartilage phenotypes cartilage tissue engineering.

Mesenchymal stem cells differentiate into the chondrocytes that form hyaline cartilaginous matrices, which serve as templates for epiphyseal growth plate formation. Since these events occur during an avascular period in a hypoxic environment, hypoxia seems to be one component of chondrogenesis regulation. Hypoxia has been reported to enhance chondrogenic differentiation in C3H10T1/2 cells by up-regulating collagen II and aggrecan and suppress hypertrophy by down-regulating collagen X via Runx2 inhibition [42]. The expansion of adipose-derived human MSCs under hypoxic conditions has also been proven to suppress hypertrophic phenotypes by downregulating Runx2 and other proteins [43]. HIF-1α, one of the major mediators of hypoxic responses, appears to be essential to chondrogenic differentiation [44] and the maintenance of cartilage phenotypes [40]. In fact, HIF-1α stabilization has become a promising method of cartilage phenotype maintenance in normoxic conditions [33]. Thus, HIF-1α could be a promising therapeutic tool for future cell-based therapy applications involving cartilage tissue engineering [40].

In this study, BMP2 was found to induce both osteogenic and chondrogenic processes in C3H10T1/2 cells in vitro. When the HIF-1α expression was upregulated during the BMP2-induced chondrogenic differentiation process in vitro, the HIF-1α potentiated BMP2-induced chondrogenesis. In addition, the Sox9 expression was significantly upregulated by HIF-1α. Sox9 a transcription factor of the sex-determining region on the Y chromosome (SRY), which regulates sex determination, chondrocyte differentiation, and numerous other developmental events [45]. During cartilage development, Sox9 is expressed in all chondroprogenitor cells; it is essential for the formation of cartilage blastema in limb mesenchyme, proliferation, chondrocyte differentiation in fetal growth plate, and the regulation of cartilage-specific genes, such as Col2A1 and aggrecan [46, 47]. Thus, Col2A1 and aggrecan, the major structural proteins of hyaline cartilage matrices, were significantly upregulated by HIF-1α as the Sox9 was upregulated. These results were consistent with our previously published data, revealing that the direct exogenous over expression of Sox9 could significantly enhance BMP2-induced chondrogenic differentiation [48].

HIF-1α also inhibited BMP2-induced osteogenesis in vitro. The expression of the Runx2 and downstream markers were significantly downregulated by HIF-1α. Runx2 is necessary for chondrocyte terminal differentiation [49], osteogenic differentiation, and bone formation during the later stages of embryonic development [28]. Thus, targeting Runx2 expression
in hypertrophic chondrocytes impairs endochondral ossification during early skeletal development [28]. Moreover, in our previous study, the over expression of Sox9 resulted in the downregulation of Runx2 expression and the inhibition of BMP-induced osteogenic differentiation and endochondral ossification. Therefore, the downregulation of Runx2 expression could be an efficient method of inhibiting chondrocyte terminal differentiation and osteogenic differentiation. Since Runx2 is essential for early chondrogenesis, which consists of mesenchymal cell condensation, proliferation, and differentiation, during the early stages of embryogenesis [50], suppressing its expression rather than completely removing it could be a more appropriate method during cartilage tissue engineering. Thus, HIF-1α satisfactorily regulated the inhibition of BMP-induced osteogenic differentiation and could adequately maintain chondrocyte phenotypes in vivo.

In this study, the regulation functions of HIF-1α during BMP2-induced osteochondrogenic differentiation were investigated via subcutaneous stem cell implantation in nude mice, and the effects of HIF-1α on the progression of BMP2-induced chondrogenesis and endochondral ossification were observed using a fetal limb culture. Through bone mass histology assay, HIF-1α was shown to inhibit the BMP2-induced formation of bone matrices, trabeculae, and endochondral ossification, resulting in the maintenance of the cartilage phenotypes in vivo. These results were consistent with the data indicating that HIF-1α inhibited BMP2-induced osteogenic differentiation in vitro. Since chondrocyte hypertrophy is one of the key physiological processes involved in the longitudinal growth of long bones, the use of hypertrophy to prevent endochondral ossification has become essential to the clinical applications of MSCs in cartilage tissue engineering [33]. In order to precisely observe the effects of HIF-1α on the BMP2-induced progression of chondrogenesis and endochondral ossification, a fetal limb culture was performed. Fetal limb cultures can mimic the progression of chondrogenesis and endochondral ossification in vitro [38, 51, 52]. The results revealed that HIF-1α and BMP2 acted synergistically in expanding the proliferating chondrocyte zone and inhibiting the BMP2-induced chondrocyte hypertrophy and endochondral ossification zone. These results were consistent with published data in that hypoxia suppressed terminal chondrocyte differentiation during endochondral ossification, during which HIF-1α was the master hypoxia regulator [42]. The results also further indicated that HIF-1α successfully inhibited BMP2-induced endochondral ossification and maintained the chondrocyte phenotypes.

Several studies have emphasized the importance of extracellular matrices (ECMs) during tissue engineering. Cell-specific ECMs have been reported to be capable of modulating the BMP2-induced osteogenic and chondrogenic differentiation of hMSCs [24]. In this study, only the effects of HIF-1α on BMP2-induced chondrogenic differentiation, osteogenic differentiation, and endochondral ossification were explored. In future studies, ECMs will be considered in order to further explore the applications of BMP2 to cartilage tissue engineering.

Conclusions

In this study, HIF-1α was systematically investigated as a regulator of BMP2-induced chondrogenic differentiation and osteogenic differentiation in vitro and in vivo, and the effects of HIF-1α on the BMP2-induced progression of chondrogenesis and endochondral ossification was explored using a fetal limb culture assay. Based on the results of these studies, HIF-1α was determined to potentiate BMP2-induced MSC chondrogenic differentiation, inhibit osteogenic differentiation and endochondral ossification, and maintain hyaline cartilage phenotypes. These findings strongly suggested that BMP2 combined with HIF-1α induced MSC differentiation could become a new method of maintaining cartilage phenotypes during cartilage tissue engineering.
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Disclosure Statement

The authors declare no conflict of interest.

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