Oxygen Tension Regulates Chondrocyte Differentiation and Function during Endochondral Ossification*

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Cartilage functions at a lower oxygen tension than most other tissues. To determine the role of oxygen tension in chondrocyte differentiation and function, we investigated the influence of oxygen tension in the pluripotent mesenchymal cell line C3H10T1/2 and 14.5E mice embryo forelimb organ culture. 10T1/2 cells and embryo forelimbs were cultured under normoxia (20% O2) or hypoxia (5% O2) in the presence of recombinant human bone morphogenetic protein 2. To elucidate the mechanism by which oxygen tension influences chondrocyte differentiation, the Smad pathway was examined using Smad6 overexpression adenovirus and Smad6 transgenic mice embryo forelimbs. The p38 MAPK pathway was examined using dominant-negative MKK3 and FR167653, a specific p38 MAPK inhibitor. The transcriptional activities of Sox9 and Runx2 were also investigated. Hypoxia promoted bone morphogenetic protein 2-induced glycosaminoglycan production and suppressed alkaline phosphatase activity and mineralization of C3H10T1/2. Thus, hypoxia promoted chondrocytic commitment rather than osteoblastic differentiation. In the mouse embryo forelimb organ culture, hypoxia increased cartilaginous matrix synthesis. These effects were primarily mediated by p38 MAPK activation, independent of Sox9. Hypoxia inhibited Col10a1 (type X collagen α1) expression via down-regulation of Runx2 activity by Smad suppression and histone deacetylase 4 activation. In conclusion, hypoxia promotes chondrocytic differentiation and cartilage matrix synthesis and suppresses terminal chondrocyte differentiation. These hypoxia-induced phenomena may act on chondrocytes to enhance and preserve their phenotype and function during chondrocyte differentiation and endochondral ossification.

A number of pathophysiological findings suggest that a correlation exists between hypoxia and chondrogenesis. For example, articular cartilage is an avascular tissue that functions at an oxygen tension that is lower than that of most other tissues. Articular cartilage derives both its nutrition and oxygen supply by diffusion from the synovial fluid and the subchondral bone. It has been estimated that articular chondrocytes in the deepest layers may have access to no more than 1–6% O2 (1–6). Furthermore, although the majority of mammalian cells derive their energy by using oxygen for mitochondrial oxidative phosphorylation (7), few mitochondria are present in articular chondrocytes (8). Carbohydrate breakdown in articular cartilage is dominated by the conversion of glucose to lactate via the Embden-Meyerhof-Parnas pathway (9–11) that consumes no O2. Similarly, during the endochondral ossification processes that occur in the growth plate, chondromodulin-1, an endogenous inhibitor of neovascularization, is highly expressed by chondrocytes. Of note, most of the growth plate is avascular (12). Recently, in an in vivo experiment, it was found that hypoxia-inducible factor 1, which appears to be one of the major regulators of the hypoxic response, is essential for chondrocyte growth arrest and survival (13). Therefore, hypoxia is considered to be a key factor for the growth and survival of chondrocytes. Chondrocytes are derived from undifferentiated mesenchymal cells that have the potential for multidirectional differentiation (14–16). Bone morphogenetic protein (BMP)2–2 promotes the chondrocytic differentiation of undifferentiated mesenchymal cells (17–21). BMP-2 activates Smad1-Smad5-Smad8, which subsequently associates with Smad4, relocated to the nucleus, and regulates the expression of target genes (22, 23). However, the influence of oxygen tension on BMP-Smad signaling remains to be elucidated. In addition to the Smad signaling pathway, p38 mitogen-activated protein kinase (p38 MAPK) is also activated by BMP-2 (24, 25). Several other cytokines (26, 27) or stress signals (28–30) can also activate the p38 MAPK pathway. Of particular interest, hypoxia has been found to be one of the stresses that can phosphorylate and activate p38 MAPK (31). Although p38 MAPK has been shown to be implicated in the regulation of chondrogenesis (32–34), the precise role of p38 MAPK in chondrogenesis remains elusive.

During endochondral ossification, chondrocytes undergo hypertrophy and secrete an extracellular matrix that becomes mineralized and allows vascular invasion and osteoblast differentiation (35, 36). To date, Runx2 and Runx3 (runt-related transcriptional factors 2 and 3) have essential roles in inducing chondrocyte hypertrophy; furthermore, these two interact closely (37–40). In addition, Runx2 is probably a direct tran-

* The abbreviations used are: BMP, bone morphogenetic protein; MAPK, mitogen-activated protein kinase; HDAC, histone deacetylase; GAG, glycosaminoglycan; WT-Smad6, wild type Smad6; DN-MKK3, dominant negative form of MKK3; ALP, alkaline phosphatase; TK, thymidine kinase; siRNA, small interfering RNA.
Oxygen Tension Regulates Chondrocytes

A-1 normoxia hypoxia

B-1

C-1 normoxia hypoxia

D-1

E-1 normoxia hypoxia

F

G normoxia hypoxia

H normoxia hypoxia
Oxygen Tension Regulates Chondrocytes

In the present study, we assessed the influence of oxygen tension on chondrocytic differentiation and cartilage matrix synthesis in the C3H10T1/2 pluripotent mesenchymal cell line and the N1511 murine chondrocyte cell line. We used the mouse embryo organ culture system with wild type mice and Smad6 transgenic mice that we had previously generated. In this paper, we show that hypoxia promotes chondrocytic commitment and cartilage matrix production via the p38 MAPK pathway but that hypoxia inhibits terminal differentiation via the Smad pathway and HDAC4 activation.

EXPERIMENTAL PROCEDURES

Cell Culture and Analysis for Chondrocytic, Osteoblastic, and Adipocytic Differentiation—C3H10T1/2 cells were obtained from RIKEN (Saitama, Japan) and were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen). Since it has been estimated that articular chondrocytes in the deepest layers may have access to no more than 1–6% O2, we selected 5% O2 as the aminated that articular chondrocytes in the deepest layers may have access to no more than 1–6% O2, we selected 5% O2 as the oxygen tension on chondrocytic differentiation and cartilage matrix synthesis in the C3H10T1/2 pluripotent mesenchymal cell line and the N1511 murine chondrocyte cell line. We used the mouse embryo organ culture system with wild type mice and Smad6 transgenic mice that we had previously generated. In this paper, we show that hypoxia promotes chondrocytic commitment and cartilage matrix production via the p38 MAPK pathway but that hypoxia inhibits terminal differentiation via the Smad pathway and HDAC4 activation.

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Oxygen Tension Regulates Chondrocytes

A

| Time after BMP stimulation | Control | Normoxia | Hypoxia |
|---------------------------|---------|----------|---------|
| 10min                     | N       | N        | H       |
| 20min                     | N       | N        | H       |
| 30min                     | N       | N        | H       |
| 60min                     | N       | N        | H       |
| P-Smad 1/5/8              |         |          |         |
| T-Smad 1/5/8              |         |          |         |

B

| Time after BMP stimulation | Control | Normoxia | Hypoxia |
|---------------------------|---------|----------|---------|
| 10min                     | N       | N        | H       |
| 20min                     | N       | N        | H       |
| 30min                     | N       | N        | H       |
| 60min                     | N       | N        | H       |
| P-p38                     |         |          |         |
| T-p38                     |         |          |         |

C-1

C-2

| O.D./µg DNA | GAG accumulation |
|------------|------------------|
| MOCK       | 0.1              |
| WT-Smad 6  | 0.5              |

D-1

D-2

| O.D./µg DNA | GAG accumulation |
|------------|------------------|
| MOCK       | 0.1              |
| DN-MKK3    | 0.5              |

E-1

E-2

| O.D./µg DNA | GAG accumulation |
|------------|------------------|
| FR (-)     | 0.1              |
| FR (+)     | 0.5              |

F-1

F-2

| O.D./µg DNA | GAG accumulation |
|------------|------------------|
| WT-Smad 6  | 0.1              |
| WT-Smad 6+FR | 0.5            |

G-1

G-2

|            | MOCK | Smad 6 | Smad 6+FR |
|------------|------|--------|-----------|
| Col2a1     |      |        |           |
| Col10a1    |      |        |           |
| 18S        |      |        |           |
Computer-assisted histological analysis for the proportion of the GAG production area per whole radius was performed using a Nikon ECLIPSE E1000 microscope with a Plan Apo objective, combined with a Nikon DXM 1200 Digital Camera (Tokyo, Japan) and WinRoof image processing software (Mitani Corp.) for Windows. Digitized pictures taken for each radius were analyzed to calculate the ratio of the area stained with safranin-O per whole radius. In situ hybridization for Col10a1 was done as described previously (53, 57). The proportional length of the hypertrophic positive signal of Col10a1 with respect to the whole hypertrophic chondrocyte zone along the midline was calculated. The care and handling of the animals and the procedures used in this study were in accordance with the guidelines of and were approved by the Osaka University Medical School Animal Care and Use Committee.

Antibodies and Reagents—Anti-phospho-Smad 1/5/8 monoclonal antibody, anti-phospho-p38 MAPK polyclonal antibody, and anti-p38 MAPK monoclonal antibody were purchased from Cell Signaling Technology (Beverly, MA). Anti-Smad 1/5 polyclonal antibody was purchased from Calbiochem. Anti-Sox9 antibody was purchased from Chemicon (Temecula, CA). Anti-HDAC4, anti-β-actin, and antiproliferating cell nuclear antigen polyclonal or monoclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Alexa Fluor® 488 goat anti-rabbit IgG (H + L) antibody was purchased from Molecular Probes, Inc. (Eugene, OR). Hoechst 33342 solution was purchased from Dojindo (Kumamoto, Japan). Recombinant human BMP-2 and a potent p38 MAPK inhibitor, FR167653, were provided by Astellas Pharmaceutical Inc. (Osaka, Japan).

DNA Constructs—Wild type Smad6 (WT-Smad6)-expressing adenovirus and the dominant negative form of MKK3 (DN-MKK3) vector were donated by Dr. Riko Nishimura (Department of Biochemistry, Osaka University Graduate School, Faculty of Dentistry). Wild type Runx2-expressing vector was donated by Dr. Toshihisa Komori (Department of Developmental and Reconstructive Medicine, Division of Oral Cytology and Cell Biology, Nagasaki University Graduate School of Biomedical Sciences).

Reporter Constructs and Luciferase Reporter Assay—Four tandems of 48-bp chondrocyte-specific enhancer segments of type II collagen α1 (Col2a1) were synthesized as previously reported (58) and inserted into the PGL3 promoter vector (Promega), 4Col2E-Luc. Six tandems of the Runx2 binding site were also inserted into the PGL3 promoter vector (Promega), 6Runx2E-Luc. Reporter assays were performed by transient transfection of 0.4 µg of the PGL3 promoter vector (4Col2E-Luc or 6Runx2E-Luc) and 0.01 µg of the TK-Renilla luciferase construct (TK Renilla) (Promega). Luciferase activity was measured using a Dual Luciferase assay kit (Promega) and luminometer (Berthold Technologies, Bad Wildbad, Germany) and normalized by determining the activity of Renilla luciferase. All experiments were performed in triplicate.

RNA Interference—RNA interference was done using commercially synthesized siRNA (Qiagen, Düsseldorf, Germany) and used as described in the protocols provided by the manufacturer. Cells were treated with siRNA to a final concentration of 10 µM. The siRNA duplex sequence targeting the HDAC4 protein was aauagucacggccaaagt (sense strands), previously described (59). Control siRNA consisted of siRNA targeted against luciferase (Dharmacon, Lafayette, CO).

Phosphorylation of Sox9—Serine/threonine phosphorylation of Sox9 was analyzed by affinity chromatography using a phosphoprotein purification kit (Qiagen). Cells were lysed using the lysis buffer provided in the kit. The extracted protein was applied to a phosphorylation purification column, and phosphorylated protein was eluted. Unphosphorylated protein was obtained from the flow-through fraction. These samples were blotted with anti-Sox9 antibody.

Western Blot Analysis—Western blot analyses were performed using whole cell lysates. To detect HDAC4 protein, nuclear extracts were obtained as previously reported (60). The MC3T3-E1 cell (mouse osteoblastic cell) was used as a control. The blots were first incubated with appropriate antibodies and then with horseradish peroxidase–coupled anti-mouse or rabbit IgG antibodies (Amersham Biosciences). For the blots, 20 µg of each sample was applied.

Northern Blot Analysis—Total RNA was isolated from C3H10T1/2 cells using the RNaseasy kit (Qiagen). The blots were hybridized with probes for Col2a1, Col10a1, Sox9, and Runx2 mRNA. 32P-Radiolabeled DNA probes were synthesized using cDNA obtained from reverse transcriptase–PCR amplification. The following primers were used: Col2a1 (forward primer, 5’-CCTGTCTGCTTCTTGTAAAAC-3’; reverse primer, 5’-AAAAATACAGAGGTGGTTGACACAGA-3’), Col10a1 (forward primer, 5’-AATCTCATAAATGGGATGGG-3’; and antisense primer, 5’-TGGCATGGATCCTGACACATC-3’).

**FIGURE 2. Hypoxia-induced GAG production and Col2a1 gene expression are mediated by the p38 MAPK pathway rather than the Smad pathway.** A and B, C3H10T1/2 cells were cultured with or without BMP-2 (500 ng/ml) under normoxia or hypoxia for 10, 20, 30, and 60 min and lysed. The cell lysates were determined by Western blotting with anti-phospho-Smad1-Smad5-Smad8 (P-Smad 1/5/8) or anti-Smad1-Smad5 (T-Smad 1/5) antibodies (A) and with anti-phospho p38 MAPK (P-p38) or anti-p38 MAPK (T-p38) antibodies (B). N, normoxia; H, hypoxia. C, F, regulatory mechanisms of GAG production by hypoxia.

Data are shown as mean ± S.E. (n = 3) (OD/µg of DNA).**, **p < 0.01; N.S., not significant. 2, regulatory mechanisms of Col2a1 and Col10a1 gene expression by hypoxia. G–I, C3H10T1/2 cells were cultured for 5 days with 500 ng/ml BMP-2 in the absence or presence of 1 µM FR167653. Northern blotting analysis for Col2a1 and Col10a1 genes was performed (10 µg of total RNA/lane). J–O, 12 h after infection with WT-Smad6 adenovirus, the cells were incubated with BMP-2 (500 ng/ml) in the absence or presence of 1 µM FR167653. LacZ expression adenovirus (MOCK) was used as a control. Northern blotting analysis for Col2a1 and Col10a1 was performed. N, normoxia; H, hypoxia.
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**Reverse Transcription PCR Analysis**—First-strand cDNA was synthesized using SuperScript II RNase H− reverse transcriptase (Invitrogen). The PCR was performed using Ex Taq (Takara Bio Inc., Otsu, Japan). The primers for the Runx2 gene were the same as those used for Northern blotting. The GAPDH primers included the forward primer (5′-CTGCTGCACCTGTGACTC-3′) and the reverse primer (5′-ACCCACCTCTCTCTGTA-3′). PCR products were purified using the PCR purification kit (Qiagen).

**Quantitative Real Time PCR Analysis**—We obtained cDNA by reverse transcription as mentioned above and proceeded with real time PCR using the Roche Applied Science Light Cycler® system. The SYBR® Green assay, in which each cDNA sample was evaluated in triplicate 20-μl reactions, was used for all target transcripts. Expression values were normalized to GAPDH. The primers for the Runx2 and GAPDH genes were the same as above. The Sox9 primers were as follows: forward, 5′-ATGAATCTCCTTGACCC-CTT-3′; reverse, 5′-TTGGGGAAGGTGTTTCTC-3′.

**Results**

**Hypoxia Promotes Chondrocytic Differentiation and GAG Production, whereas It Suppresses Osteoblastic Differentiation and Chondrocyte Terminal Differentiation in C3H10T1/2 Cell Culture**—In C3H10T1/2 cell culture, BMP-2 induced GAG production in a dose-dependent manner (Fig. 1A). At every BMP-2 concentration tested, hypoxia clearly increased GAG content additively with BMP-2. Hypoxia-induced GAG synthesis was also found in the N1511 murine chondrocyte culture (Fig. 1B). On the other hand, low oxygen tension clearly suppressed ALP activity and alizarin red staining of C3H10T1/2 cells (Fig. 1C and D), suggesting that hypoxia suppressed BMP-2-induced osteoblastic differentiation. Adipocytic differentiation, as identified by oil red O staining, was not affected by hypoxia.
oxygen tension (Fig. 1E). After cell density became confluent, there was no difference of the cell proliferation between normoxia and hypoxia at 7 days in C3H10T1/2 cells and N1511 cells (Fig. 1F). The type II collagen (Col2a1) gene, a well characterized, specific marker of commitment to the chondrogenic lineage, was up-regulated by BMP-2 in a dose-dependent manner (Fig. 1G) as well as by hypoxia at each BMP-2 concentration tested. The mRNA expression of the type X collagen (Col10a1) gene, a well characterized, specific marker for chondrocyte terminal differentiation (61, 62), was suppressed by hypoxia (Fig. 1H), in contrast to Col2a1 mRNA expression.

**Hypoxia-induced GAG Production Is Mediated by the p38 MAPK Pathway rather than the Smad Pathway**—To investigate the intracellular signal transduction mechanisms responsible for hypoxia-induced chondrocyte differentiation, we first examined the activities of the Smad and the p38 MAPK pathways. As shown in Fig. 2, A and B, phosphorylation of p38 MAPK is up-regulated by hypoxia, whereas phosphorylation of Smad is down-regulated. When Smad signaling was inhibited by the overexpression of WT-Smad6, which inhibits phosphorylation of the Smad1-Smad5-Smad8 complex, BMP-2-induced GAG production was markedly reduced under both oxygen levels. However, hypoxia was still able to promote GAG production despite Smad inhibition (Fig. 2C). In contrast, hypoxia-induced GAG production was abolished by the overexpression of DN-MKK3 that specifically inhibits p38 MAPK phosphorylation (Fig. 2D) and also by FR167653, a specific p38 MAPK inhibitor (Fig. 2E). When both the Smad and the p38 MAPK pathways were blocked by Smad6 overexpression and FR167653, hypoxia did not influence the GAG production level (Fig. 2F).

**Regulatory Mechanisms of Col2a1 and Col10a1 Gene Expression by Oxygen Tension**—Next, we assessed the role of the p38 MAPK and Smad pathways in the regulation of Col2a1 and Col10a1 gene expression. In the presence of FR167653, hypoxia-induced Col2a1 expression was suppressed (Fig. 2G-1). However, when WT-Smad6 was overexpressed, although Col2a1 gene expression was suppressed under both oxygen conditions, hypoxia strongly induced the Col2a1 gene. When both Smad and p38 MAPK signaling was blocked by WT-Smad6 and FR167653, Col2a1 gene induction caused by hypoxia was again strongly attenuated (Fig. 2G-2). Our findings suggest that hypoxic regulation of Col2a1 gene expression is mediated by the p38 MAPK pathway rather than the Smad pathway, which is very similar to the mechanism of hypoxic regulation involved in GAG production. On the other hand,
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A-1

| normoxia | hypoxia |
|----------|---------|
| Sox9     | Sox9    |
| 18S      | 18S     |

A-2

N.S.

proportion ratio (Sox9/GAPDH)

normoxia hypoxia

C

control normoxia hypoxia

phospho- Sox9 un-phospho-Sox9

B

RCS cells/ 4Col2E-luc

N.S.

4SoxE-Luc

pGL3*

relative luciferase activity (RLU)

hypoxia

hypoxia

C3H10T1/2 cells/ 4Col2E-luc

BMP(-) BMP(500ng/ml)

N.S.

N.S.

D-1

MOCK MOCK +FR Smad6

N H N H N H

Runx2 18S

D-2

proportion ratio (Runx2/GAPDH)

MOCK Smad6 Smad6+FR

N.S. N.S. N.S.

F

WT-Runx2 WT-Runx2 (-) WT-Runx2 (+)

normoxia hypoxia hypoxia

MC3T3-E1 cells / 6Runx2E-luc

C3H10T1/2 cells / 6Runx2E-luc

WT-Runx2 - + - +

** relative luciferase activity (RLU)

GAPDH

18S

Col10a1

Runx2
Col10a1 gene expression was reduced by hypoxia as shown in Fig. 1G, and it seemed to be regulated in a complicated manner that is different from Col2a1 gene expression. The blockade of the p38 MAPK pathway did not alter the hypoxia-related reduction of Col10a1 gene expression, but the blockade of the Smad pathway abolished it. However, when both pathways were blocked, hypoxia reduced Col10a1 gene expression (Fig. 2G).

**Hypoxia Enlarges the Cartilaginous Matrix Area Associated with Endochondral Ossification via the p38 MAPK Pathway in Organ Culture**—Our data show that hypoxia promoted the commitment of C3H10T1/2 cells to a chondrocytic lineage and enhanced cartilage matrix production via the p38 MAPK pathway. To further confirm the influence of oxygen tension on cartilage biology in a setting similar to that found in vivo, we cultured embryonic day 14.5 embryo forelimb organs obtained from wild type and Smad6 transgenic mice. In ex vivo embryo limb cultures in the presence of BMP-2, explants grew quickly both in length and in width and wound greatly as they grow. Therefore, to evaluate cartilaginous matrix production, we calculated the proportion of the area of each radius that was stained with safranin-O compared with the whole radius area. The embryo forelimbs that were cultured under hypoxic conditions showed a significantly greater enlargement in the matrix area, and hypoxia-induced cartilage enlargement was abolished by FR167653 (Fig. 3, A–1). In explants from Smad6 transgenic mice, hypoxia enlarged the cartilage matrix area, and FR167653 abolished hypoxia-induced matrix enlargement (Fig. 3, B–1). These findings are consistent with our *in vitro* observation that hypoxia promotes cartilaginous matrix synthesis not via the Smad pathway but via p38 MAPK signaling (Fig. 2G).

**Terminal Differentiation of Chondrocyte Is Suppressed by Hypoxia in Mouse Embryo Organ Cultures**—In C3H10T1/2 cell cultures, Col10a1 gene expression was repressed by hypoxia. In accordance with this, when using *in situ* hybridization, we found that, in wild type embryo forelimb cultures, hypoxia reduced the Col10a1 gene expression level estimated by the proportional length of the Col10a1-positive site per whole hypertrophic chondrocyte zone (Fig. 4, bidirectional arrows) along the midline. When p38 MAPK signaling was suppressed using FR167653, the Col10a1-positive area was markedly reduced with both oxygen levels. In addition, hypoxia apparently reduced the Col10a1-positive area regardless of the use of p38 MAPK inhibitor (Fig. 4A). On the other hand, hypoxia alone did not repress Col10a1 gene expression in Smad6 transgenic mice embryo forelimb organ culture, although hypoxia did suppress Col10a1 gene expression when the p38 MAPK pathway was inhibited by FR167653 (Fig. 4B). These findings were in agreement with the *in vitro* results and suggest that the Smad pathway mediates Col10a1 gene down-regulation and that the p38 MAPK pathway mediates its up-regulation. In addition, hypoxia reduced Col10a1 expression even when both Smad and p38 MAPK signaling were blocked, which would suggest that unknown factors that down-regulate Col10a1 gene expression are involved.

Sox9 Gene Expression and Its Transcriptional Activity Are Not Up-regulated by Hypoxia—Sox9 is a key transcriptional factor for chondrocytic differentiation and regulates transcription of the Col2a1 gene. Thus, we evaluated the influence of oxygen tension on Sox9 gene expression. Interestingly, Sox9 mRNA expression was not altered by hypoxia (Fig. 5A). As shown in Fig. 5B, Sox9 transcriptional activity was clearly up-regulated by BMP-2. However, it was not up-regulated but rather was down-regulated by hypoxia. Sox9 transcriptional activity has been reported to be enhanced by cAMP-dependent protein kinase A serine/threonine phosphorylation (63). We found that phosphorylation of Sox9 was not promoted but was suppressed by hypoxia (Fig. 5C), which was consistent with the results of the reporter assay.

Runx2 Gene Expression and Its Transcriptional Activity Are Suppressed by Hypoxia—Since hypoxia suppressed chondrocyte hypertrophy and osteoblastic differentiation, we studied the role of Runx2 in the regulation of hypoxia-induced phenomena. Hypoxia suppressed Runx2 gene expression and its transcriptional activity in C3H10T1/2 cells stimulated with BMP-2 (Fig. 5, D and E). Blockade of the p38 MAPK pathway did not alter the suppression of Runx2 caused by hypoxia, but blockade of the Smad pathway abolished the effect of hypoxia. However, when both pathways were blocked, hypoxia could again suppress Runx2 gene expression (Fig. 5D). This pattern is very similar to the regulatory pattern we found for the hypoxic regulation of Col10a1 gene expression. To examine how Runx2 contributes to chondrocyte terminal differentiation in hypoxia, we transfected C3H10T1/2 cells with vector expressing wild type Runx2. When Runx2 was overexpressed, the Runx2 transcriptional activity suppressed by hypoxia was remarkably up-regulated to a level comparable with that with normoxia (Fig. 5E). In addition, Col10a1 gene expression that had been reduced by hypoxia recovered to a level that matched the level expressed under normoxic conditions with Runx2 overexpres-
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A

HDAC4 (western blot analysis)

N1511  C3H10T1/2  MC3T3-E1
nucleus

HDAC 4

cytoplasm

β actin

B-1

proliferative chondrocyte
hypertrophic chondrocyte

B-2

HDAC4 (immunohistochemical analysis)

C-1

hoechst  HDAC4  Merge

normoxia

hypoxia

C-2

N  H

nucleus

HDAC 4

PCNA

cytoplasm

HDAC 4

β actin

D

MOCK  siRNA HDAC4

HDAC4

PCNA

Runx2

GAPDH

E

normoxia  hypoxia

Runx2  Col10a1

18S

(day2)

(day5)

F

C3H10T1/2 cells

6Runx2E-luc

relative luciferase activity (RLU)

normoxia  hypoxia

siRNA control  siRNA HDAC4

*  N.S
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Murine mesenchymal C3H10T1/2 cells are pluripotent and differentiate into several lineages (19). BMP-2 and BMP-7 induce C3H10T1/2 cells to differentiate into osteoblasts, chondrocytes, and adipocytes; a low concentration favors adipocytes, and a high concentration favors chondrocytes and osteoblasts (18, 19, 64, 65). Thus, C3H10T1/2 cells are an appropriate model for studying the mechanisms of pluripotent mesenchymal cell commitment into a particular lineage. In the present study, we cultivated C3H10T1/2 cells in the presence or absence of recombinant human BMP-2 under normoxia or hypoxia. BMP-2 treatment and low oxygen tension synergistically induced GAG production and Col2a1 gene expression and profoundly suppressed ALP activity and mineralization; they did not alter fat droplet production. These data indicate that hypoxia promoted chondrocytic commitment of the pluripotent C3H10T1/2 mesenchymal cells and inhibited osteoblastic differentiation. Our results are in accordance with previous reports, which describe that hypoxia enhances Col2a1 and Aggrecan gene expression in C3H10T1/2 cells (66).

To further investigate the role of oxygen tension in chondrocytic differentiation, we used a mouse embryo forelimb explant culture system. This system enables one to cultivate cartilage tissue under hypoxic conditions for up to 2 weeks without any interference from systemic, hormonal, or neuronal responses to the hypoxia that could affect cartilage metabolism while at the same time examining the effects of both environmental factors and cytokines on the endochondral ossification process. In organ cultures of wild type mice forelimbs at 14.5 days postcoitum, we found that hypoxia clearly induced the enlargement of the cartilage matrix area stained by Safranin-O, suggesting that hypoxia promoted cartilage matrix synthesis by chondrocytes during endochondral ossification. To confirm this, we cultured N1511 chondrocytes under hypoxic conditions and found that hypoxia induced N1511 cells to produce GAG. Our data indicate that hypoxia not only induced the commitment of pluripotent mesenchymal progenitors into a chondrocyte lineage but also activated the production by chondrocytes of cartilage matrix.

In the present study, hypoxia in the presence of BMP-2 clearly suppressed Col10a1 mRNA expression in C3H10T1/2 cell culture and also in organ culture studies using tissues from wild type animals. This suggests that hypoxia suppresses the terminal differentiation of chondrocytes during endochondral...
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ossification. Thus, hypoxia seems to act on chondrocytes to preserve their chondrocyte phenotype by preventing hypertrophy and, consequently, terminal differentiation.

To confirm the mechanisms by which these hypoxia-induced phenomena operate, we studied the signaling pathways of BMP-2 that are well known to induce chondrogenesis of mesenchymal cells (17–21) via the induction of Sox9 gene expression (67). Basically, BMP-2 signals propagate through the Smad pathway and bind directly or via other DNA-binding proteins to the promoters of BMP-2-responsive genes to stimulate or repress their transcription. Over the past few years, evidence has accumulated that suggests that BMP-2 may also stimulate other downstream pathways involving p38 MAPK. In the present study, we found that hypoxia enhanced BMP-2-induced activation of the p38 MAPK pathway, whereas hypoxia did not promote and could even suppress the Smad pathway. Furthermore, our Smad- and p38 MAPK-suppressing studies using WT-Smad6, DN-MKK3, and FR167653 revealed that it is not the Smad pathway but the p38 MAPK pathway that is indispensable for hypoxia-induced cartilaginous matrix synthesis. Overall, our data indicate that hypoxia promotes cartilaginous matrix synthesis via the p38 MAPK pathway.

Little is known about how oxygen tension regulates the signal transduction systems that regulate Col10a1 gene expression. In the present study, we found that hypoxia promoted BMP-2-induced activation of the p38 MAPK pathway but did not promote, and could even suppress, the Smad pathway. Previous reports suggested that Smad1-Smad5 signaling positively regulates type X collagen expression by potentiating the transcriptional activity of Runx2 (44, 45) and that p38 MAPK signaling also promotes its expression (34, 45, 68) via Runx2 activation. These observations are consistent with the results of our experiments that blocked these pathways by Smad6, DN-MKK3, or the p38 inhibitor, FR167653. This suggests that hypoxia positively regulates Col10a1 gene expression via the up-regulation of p38 MAPK signaling and negatively regulates Col10a1 gene expression via down-regulation of the Smad pathway. In addition to this, we found that hypoxia up-regulated HDAC4 activity, which recently was reported to control chondrocyte hypertrophy by suppressing Runx2 gene expression through inhibition of the gene’s positive feedback mechanism and the suppression of its transcriptional activity (47). We were able to confirm that hypoxic suppression of Runx2 gene expression, transcriptional activity, and Col10a1 gene expression were blocked by silencing HDAC4. This indicates that hypoxia activates HDAC4, thereby suppressing Runx2 activity, which in turn down-regulates Col10a1 gene expression. Our data reveal that hypoxia inhibits the hypertrophy of chondrocytes by down-regulating Runx2 activity based on the sum of the positive regulation that occurs via p38 MAPK activation and the negative regulation that is caused by Smad signaling suppression and HDAC4 activation.

Transcriptional factors Sox9, L-Sox5, and Sox6, have been reported to be essential and sufficient for regulating the expression of Col2a1 and the other genes involved in the chondrocytic program (69). This would suggest that Sox transcriptional factors should be up-regulated by hypoxia and thus propagate the signals for chondrocytic differentiation and cartilage matrix production. However, very interestingly, our results indicate that hypoxia-mediated chondrocytic differentiation did not involve the up-regulation of Sox9 gene expression, its phosphorylation, or its transcriptional activity, suggesting that hypoxia-induced Col2a1 induction was independent of Sox9. However, in the previous report, it was described that Sox9 gene expression was up-regulated by hypoxia in association with transactivation of the Sox9 promoter in ST2 stromal cells (66). Although the difference in the results might depend on the difference in cell type, further confirmation about the Sox9 promoter may be necessary. The results of the reporter assay lead us to speculate that transcription factors other than Sox9 directly regulate Col2a1 gene expression via other cis elements located at a site other than the chondrocyte-specific enhancer fragment of the Col2a1 gene (69, 70). The Sox trio shares a cis element in the Col2a1 chondrocyte-specific enhancer region; however, to exclude the involvement of L-Sox5 and Sox6 in hypoxia-induced Col2a1 gene expression, further confirmation, including gene expression of these transcription factors, is needed. It is known that hypoxic stress reduces protein kinase A activity (71). Recently, protein kinase A was reported to phosphorylate Sox9 and to enhance its transcriptional activity (62). Taking these data into account, it is plausible that Sox9 transcriptional activity could have been down-regulated by hypoxia. However, the actual transcription factors responsible for hypoxia-induced Col2a1 induction remain to be elucidated (Fig. 7).

In conclusion, we demonstrated that hypoxia clearly promoted chondrocytic commitment of cells in the mesenchymal lineage, as well as cartilaginous matrix synthesis, and inhibited terminal differentiation both in cell culture and organ culture. These effects were primarily mediated by p38 MAPK activation.
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independent of Sox9. On the other hand, hypoxia inhibited the hypertrophy of chondrocytes via the down-regulation of Runx2 activity through Smad signaling suppression and HDAC4 activation. These hypoxia-induced phenomena affect mesenchymal cell differentiation and endochondral ossification by enhancing and preserving the chondrocytic phenotype and cell function, as well as preventing chondrocytes from terminal differentiation that subsequently leads to matrix degeneration and chondrocyte apoptosis. The hypoxia-associated regulation of chondrocytes that has been outlined in our study may be of fundamental importance in the biology and pathology of cartilage tissues and may be involved in endochondral ossification in the growth plate, in joint cartilage homeostasis, and in the etiology of osteoarthritis.

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