

Hypoxia induces chondrocyte-specific gene expression in mesenchymal cells in association with transcriptional activation of Sox9

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Abstract

Endochondral bone is formed during an avascular period in an environment of low oxygen. Under these conditions, pluripotential mesenchymal stromal cells preferentially differentiate into chondrocytes and form cartilage. In this study, we investigated the hypothesis that oxygen tension modulates bone mesenchymal cell fate by altering the expression of genes that function to promote chondrogenesis. Microarray of RNA samples from ST2 cells revealed significant changes in 728 array elements ($P < 0.01$) in response to hypoxia. Real-time PCR on these RNA samples, and separate samples from C3H10T1/2 cells, revealed hypoxia-induced changes in the expression of additional genes known to be expressed by chondrocytes including Sox9 and its downstream targets aggrecan and Col2a. These changes were accompanied by the accumulation of mucopolysaccharide as detected by alcian blue staining. To investigate the mechanisms responsible for upregulation of Sox9 by hypoxia, we determined the effect of hypoxia on HIF-1 α levels and Sox9 promoter activity in ST2 cells. Hypoxia increased nuclear accumulation of HIF-1 α and activated the Sox9 promoter. The ability of hypoxia to transactivate the Sox9 promoter was virtually abolished by deletion of HIF-1 α consensus sites within the proximal promoter. These findings suggest that hypoxia promotes the differentiation of mesenchymal cells along a chondrocyte pathway in part by activating Sox-9 via a HIF-1 α -dependent mechanism.

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Introduction

The ability of cells to sense and respond to changes in oxygen (O₂) tension is critical for many developmental, physiological, and pathological processes, including angiogenesis, control of blood flow, and tissue repair. In many tissues including bone, hypoxic stress induces the expression of genes whose products act in concert to facilitate the supply of metabolic energy [1–3]. For example, hypoxia

induces vascular endothelial growth factor (VEGF), which functions to promote angiogenesis during development and following injury. A number of oxygen-sensitive genes including *vegf* are known to be under transcriptional control by hypoxia-inducible factors (HIFs). HIFs activate transcription by binding to the hypoxia-responsive elements (HRE) in the proximal promoter region of oxygen-responsive genes [4].

During endochondral bone formation, mesenchymal stem cells differentiate into chondrocytes that form a hyaline cartilaginous matrix which serves as a template for formation of the epiphyseal growth plate. These events occur during an avascular period in a hypoxic environment

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[5]. As bone is vascularized and the tissue becomes oxygenated, osteoblasts arise from the bone marrow mesenchyme and begin to deposit and mineralize osteoid along the cartilage matrix. Interestingly, the cellular events that occur following fracture largely recapitulate those that occur during endochondral bone formation [6]. Thus, disruption of vascular supply to the fracture site, and consequent hypoxia, results in the appearance of chondrocytes and formation of a cartilaginous callous. This is followed by vascular in-growth and the appearance of osteoclasts and osteoblasts which remodel the cartilaginous callous to form mineralized bone. Since chondrocytes and osteoblasts are believed to originate from a common stromal stem cell [7–10], it has been suggested that the local tissue level of O₂ might influence the developmental fate of bone progenitor cells [11,12].

The molecular mechanisms that control the commitment of mesenchymal stromal cells into the chondrocyte lineage are not fully understood. The early events appear to involve the coordinated activities of transcription factors downstream of BMP signaling [13] and members of the Sox9 High Mobility Group superfamily [14]. Sox9 is expressed in early mesenchymal condensations and is induced by BMPs, whereas noggin, a BMP antagonist, blocks expression of Sox9 [15]. Once committed to this pathway, chondrocytes then undergo an orderly process of differentiation which is regulated by a complex series of signaling molecules and transcription factors including Runx2/3, parathyroid hormone-related protein, Ihh, and Wnts [16].

In this study, we used microarray analysis in combination with real-time PCR to identify genes that are regulated by hypoxia in mesenchymal stromal ST2 and C3H10T1/2 cells. We show that hypoxia upregulated genes associated with chondrocyte differentiation and induced phenotypic changes consistent with chondrocyte lineage progression. Importantly, hypoxia transcriptionally activated Sox9, a key transcription factor required for chondrogenesis. These findings suggest that hypoxia is an early event that participates in cartilage progenitor cell gene programming.

Materials and methods

Cell culture and RNA extraction

Tissue culture supplies were purchased from Fisher Scientific (Pittsburgh, PA). Mouse ST2 stromal cells [17], immortalized from murine bone marrow (originally from Riken Cell Bank, Tsukuba Science City, Japan), were a generous gift from Dr. Janet Rubin (Emory University, Atlanta, GA). ST2 cells were maintained in α -MEM containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 μ g/ml) in a water-jacketed incubator with a humidified atmosphere (5% CO₂/air) at 37°C. C3H10T1/2 cells [18] were purchased from American Type Tissue Collection and maintained in DMEM containing 10% FBS.

Medium was changed every 48 h, and cells were passaged when they reached 95% confluence. For the hypoxia studies, cells were grown in 100-mm tissue culture plates to approximately 75% confluence and then incubated in 1% O₂ or 21% O₂ in an incubator configured to deliver regulated levels of oxygen (Forma Sci, Marietta, OH). At each time point, total RNA was extracted using the RNA STAT 60 reagent (Tel-Test, Gainesville, FL) in accordance with the manufacturer's instructions. The quality and quantity of the RNA were assessed with the Agilent 2100 bioanalyzer (Agilent Biotechnologies, Wilmington, DE).

DNA microarray

The microarray was constructed by the Genomics and Microarray Laboratory, in the Center for Environmental Genetics at the University of Cincinnati (<http://microarray.uc.edu/>) using the Incyte Genomics mouse GEM1 clones amplified by PCR and printed onto glass slides (Omnigridd Microarrayer; GeneMachines, San Carlos, CA). Based on current mouse genome annotations, this set of 8734 cDNAs contains 3205 clones for known genes, 2045 for RIKEN cDNAs, 2103 nonannotated expressed sequenced tags (ESTs), 1066 annotated ESTs, and 315 DNA segments or hypothetical proteins. Using structural or literature-based extraction of features of predicted or known proteins, 3991 of these are classifiable by molecular function, biological process, or cellular component. Each sample (20 μ g) of RNA was reverse transcribed and tagged with fluorescent Cy5 dye. A universal reference was prepared with pooled RNA obtained from each of the experimental time points, reverse transcribed, and tagged with fluorescent Cy3 dye. Cy3 and Cy5 samples were then competitively hybridized to the microarrays, washed, and scanned at 635 (Cy5) and 532 (Cy3) nm (GenePix 4000B; Axon Instruments, Inc., Union City, CA). Defective spots (i.e., irregular geometry, scratched, or containing artifact) were eliminated using the GenePix Pro software (Axon Instruments, Union City, CA) and by manual inspection.

Real-time RT-PCR

Synthesis of cDNA was performed from 5 μ g of mRNA using the Superscript first strand synthesis kit (Invitrogen, Carlsbad, CA). Forward and reverse primers were designed to span a single intron to generate a product of 100–120 base pairs with a T_m of approximately 58°C. The primer sequences were as follows: CTGF: sense 5' GGGCTGATGCAGAAGTTCCTTCG-3' antisense 5'-GCAATTTCCTGGCGCTGAGC-3', C4ST2: sense 5'-CACAC-TGACATGCCCAAGAC-3', antisense 5'-CCTTTCCTTCTCCTTTTGCATG-3', ANX5: sense 5'-GCAA-TTTCCTGGCGCTGAGC-3' antisense 5'-GGGCTGATGCACAAGTCCTTCG-3', LOX: sense 5'TCTATGTCTGCCGCATAGGTG-3' antisense 5'-GGAGGACACGTCCTGTGAC-3', Sox9: sense 5'-TTCCTCTC-

CCGGCATGAGTG-3' antisense 5'-CAACTTTGCCAGCT-TGCACG-3', Aggreca: sense 5'-GCGTGAGCATCCCT-CAACCATC-3' antisense 5'-GGCAGTGGTCACAGGATG-CATG-3', Col2a sense 5'-AAGGTGCTCAAGTTCTCGTG-3' antisense 5'-TTTGGCTCCAGGAATACCATC-3', β -actin: sense 5'-CTGAACCCTAAGGCCAACCCTG-3' antisense 5'-GGCATAACAGGGACAGCACAGCC-3'.

PCR reactions were carried out in 25 μ l final volume containing 12.5 μ l of 2 \times SYBR green master mix (Qiagen, Valencia, CA), 200 nM of each primer, 9 μ l of RNase-free water, and 1 μ l of cDNA. PCR reactions were performed on the Smart Cycler thermal cycler (Cepheid, Sunnydale, CA). Predicted cycle thresholds were calculated using Smart Cycler software, and a melting curve was produced by slow denaturation of the PCR products to validate the specificity of amplification. Cycle thresholds were normalized to β -actin to control for cDNA quantification differences. Data were analyzed using the Q-Gen software for real-time PCR [19].

Western blot analysis

Nuclear extracts (50 μ g) were boiled for 5 min in Laemmli buffer [62.5 mM Tris (pH 6.8), 1% SDS, 20% glycerol, 0.01% bromophenol blue, and 100 mM DTT] and separated on 6% SDS-PAGE gels. Gels were then transferred to 0.2 μ m nitrocellulose membranes. After blocking with TBS-T [TBS (pH 7.4) and 0.1% Tween-20] containing 5% low fat milk, the membranes were incubated with primary antibody overnight in blocking buffer followed by horseradish-peroxidase-conjugated secondary antibody for 2 h and developed by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). Analysis of HIF-1 α and HIF-1 β was performed on evenly loaded immunoblots by sequential reprobing with each antibody after stripping using 2% SDS, 62.5 mM Tris pH 6.7, 100 mM mercaptoethanol for 30 min at 50°C. Antibodies against HIF-1 α and HIF-1 β were from NeoMarkers (Freemont, CA) and Novus Biologicals (Littleton, CO), respectively.

Preparation of Sox9 promoter constructs

The wild-type Sox9 promoter (Kpa Sox9) encoding a 6.8 kb fragment (−6.8 kb to +251 bp and ending at +315 bp relative to the transcription start site of the 5' flank), and several deletion fragments were cloned upstream of a firefly luciferase cDNA [20]. Mutant constructs encoding a 3-nt substitution (GAAAG) in this core sequence GCGTG were prepared in the context of the full-length Sox9 promoter by PCR using the following primers: Mutant 1 forward primer (93 bp): ATAGGTACCACGGAGACAGCATCGAAAAGTGGGGGTGGGGGGTTGTGGAGGGTCTAGTCTAGACACGCTCGAAAGCACGCGCACACACACAC 3'; Mutant 2 forward primer (87 bp) ATAGGTACCACGGAGACAGCATCGAAAAGTGGGGGTGGGGGGTTGTGGAGGGTCTAGTCTAGACTTTCTCG-

CGTGCACGCGCACAC; Mutant 3 forward primer ATAGGTACCACGGAGACAGCATCGAAAAGTGGGGGTGGGGGGTTGTGGAGGGTCTAGTCTAGACACGCTCGCGTGCTTTTCGCACACACACACACA; Reverse primer: (30 bp) 5'-GGAGGGGAGCTCAGCCAGAGGGTGGATGGT-3' shown in Table 1. PCR was performed under the following conditions: 2 min at 94°C; 0.3 min at 94°C; 0.5 min at 61°C; 0.5 min at 72°C for 33 cycles; and 5 min at 72°C. The Kpa Sox9 plasmid was then digested with *Kpn* and *SSTI*, ligated into the luciferase expression plasmid, purified (Qiagen, Valencia, CA), and then sequenced.

Transient transfection assays

The Sox9 promoter-luciferase constructs and a *KpnI*–*NheI* fragment comprising of −2273 to +51 bp of VEGF promoter fused to luciferase (Dr. J. Abraham, Scios Inc, CA) were purified using commercial kits (Qiagen, Valencia, CA). ST2 cells were cultured to 60–80% confluence in 12-well plates. For each well, 1.6 μ g of plasmid DNA and 4 μ l of lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) were diluted separately in 50 μ l of OPTI-MEM I reduced serum medium (Invitrogen, Carlsbad, CA), mixed together, and incubated at room temperature for 30 min. Plates were then washed with serum-free medium, and 0.4 ml of OPTI-MEM I reduced serum medium was added, and the diluted solution was added to the cells. Plates were incubated at 37°C for 5 h after which time growth medium containing 20% serum was added. Cells were allowed to recover under normoxic conditions for 19 h and then exposed to 21% or 1% O₂ for 24 h. Cells were collected, lysed, and luciferase

Table 1
Categorical distribution of ST2 gene array elements significantly altered by hypoxia

	Induced	Biphasic	Suppressed	Total
Cell cycle	1	6	6	13
Inflammatory mediator	3	5	7	15
Signal transduction	16	14	5	35
Membrane receptor	5	5	1	11
Transcription	12	18	13	43
Translation	8	17	3	28
Metabolism	17	20	7	44
Stress response	3	3	0	6
Inflammatory response	2	4	6	12
Apoptosis	4	3	1	8
Transporter	8	11	2	21
DNA	3	1	1	5
Angiogenesis	8	0	0	8
Keratin	1	2	2	5
Cytoskeleton	8	9	4	21
Extracellular	4	1	2	7
Cell adhesion	4	1	1	6
Membrane protein	10	7	2	19
Proteolysis	6	6	2	14
EST	114	169	116	399
Totals	236	302	181	719

assays were carried out using the Steady-Glo luciferase assay system (Promega). The relative luciferase activity (mean \pm standard error of the mean [SEM]) was calculated as light units/ μ g protein. All experiments were repeated at least three times with two different batches of purified DNA. The protein concentration was measured using the Coomassie Plus protein assay reagent (Pierce Chemical, Rockford, IL).

Mucopolysaccharide staining

ST2 cells were plated in six-well culture dishes at high density and then treated with 21% or 1% oxygen for 7 days. In a parallel set of experiments, C3H10T1/2 cells were treated with 21% or 1% oxygen for up to 16 days. In both experiments, culture media was changed every 48 h. Cells were fixed for 10 min with 95% ethanol and stained with alcian blue overnight as described [21].

Statistics

Primary data from the microarray were examined using GenePix software (Axon Instruments, Inc.) and GeneSpring 4.2 software (Silicon Genetics, Redwood City, CA). Data sets were subjected to normalization within each microarray experiment such that the median of the Cy5 channel was balanced against the ratio of the Cy3 channel followed by an intensity-dependent Lowess normalization. Each microarray contained control non-mammalian single gene spikes to ensure the validity of interarray comparisons. Gene expression in the control and experimental groups was compared using Student's *t* test; a *P* value < 0.01 was assigned as significant. Pools of genes that were differentially expressed over time were clustered according to their expression pattern dynamics into hierarchical tree clustering algorithms using a minimum distance value of 0.001, a separation ratio of 0.5, and the Pearson's correlation distance definition. Gene sets with poor internal consistency were eliminated. Mean expression data from the real-time PCR were analyzed using ANOVA and Tukey's posttest analysis with a *P* value of < 0.05 considered significant. Comparisons between the slopes of the microarray and the real-time PCR data were made with linear regression analysis, using SAS software, and were considered similar with a *P* value of > 0.05 .

Results

Global changes in ST2 cell gene expression in response to hypoxia

To examine the gene expression profiles of ST2 cells exposed to hypoxia, microarray analysis was performed, and RNA samples were collected at time zero and 1, 3, 6,

12, 24, and 48 h after exposure to 1% or 21% oxygen. For each condition, two independent cultures were used, and each one was subjected to independent RNA isolation, labeling, and GeneChip hybridization. GeneSpring software was then used to impose a series of normalization and filtering criteria to identify a list of genes reproducibly responsive to hypoxia. Pearson's correlation performed on data at each time point revealed 729 genes whose expression was significantly ($P < 0.01$) affected by hypoxia. Of these genes, 150 genes were noted to change by greater than 2-fold when compared to control. To characterize the patterns of change in gene expression associated with time of exposure to hypoxia, the Cy5/Cy3 expression ratios from the 729 genes were subjected to hierarchical tree clustering. The majority of genes exhibited induction or repression over multiple time points, thereby decreasing the likelihood that the changes in gene expression were artifact. This analysis revealed that 233 genes were upregulated, 181 genes were repressed, and 305 genes showed biphasic behavior. The patterns of gene expression in each cluster followed relatively simple kinetic patterns without marked fluctuations between adjacent time points, which suggested a low noise component.

The genes were further sub-grouped into categories according to their ascribed functions (Table 1). These categories included genes involved in signal transduction (35 genes), metabolism (44 genes), angiogenesis (8 genes), and expressed sequence tags (ESTs) or genes of unknown function (399 genes). As expected, a number of gene products known to be induced by hypoxia were identified including: hexokinase [22,23], GAPDH [24], glycogen synthase [25,26], caspase [27–29], and the EST hypoxia-induced gene 1 [30]. To further validate the temporal pattern of gene expression observed in the microarray, ST2 cells were exposed to 1% or 21% oxygen for a time course identical to that performed for the microarray, and the changes in mRNA expression of several genes were quantified by real-time PCR. Comparison of the time course of changes in the expression of each of these genes by linear regression analysis demonstrated that they were statistically similar ($P > 0.05$) to the time course plots obtained in the microarray. The entire gene expression data and cluster groups can be accessed at: <http://genet.cchmc.org> (username: hypoxia, password: hypoxia).

Hypoxia induces genes expressed by chondrocytes

The patterns of expression of ten genes known to be expressed by chondrocytes were significantly altered by hypoxia (Table 2). Chondroitin-4-sulfonotransferase-2, an enzyme necessary for the formation of the mucopolysaccharide component of cartilage [31,32], was induced 6-fold. Connective tissue growth factor (CTGF), a secreted protein that mediates interactions with growth factors,

Table 2
Changes in expression of selected genes associated with chondrocyte differentiation

Gene name	Genbank accession number	Fold change
Chondroitin-4-sulfonotransferase 2	NM_021528	+6
Connective tissue growth factor	NM_010217	+6
Annexin 2	NM_007585	+6
Annexin 5	NM_009673	+6
Lectin galactose binding protein 3	BI414633	+6
Lysyl oxidase	NM_010728	+3
Beta-1,3-glucuronyltransferase 3	NM_024256	+2
GL1-Kruppel family gene	NM_010296	+1.9
Twisted gastrulation protein	BC004850	-1.8
Laminin receptor	NG_001500	+1.6

integrins, and extracellular matrix components, required for cell proliferation and matrix remodeling during chondrogenesis [33–35], was induced 6-fold. The annexins are calcium binding proteins that have been shown to regulate the maturation of the cartilaginous growth plate [36–39]. Both annexins 2 and 5 were induced 6-fold over the course of the experiment. The expression of lectin galactose binding protein-3, a desialylated glycoprotein, which is expressed by differentiated chondrocytes [40,41], was induced 6-fold. The copper enzyme lysyl oxidase, which plays a role in initiation of collagen crosslinking, was increased 3-fold [42]. Two genes were identified that

have been shown to regulate bone morphogenic protein (BMP) expression, a critical morphogen involved in both osteoblast and chondrocyte differentiation [43–45]. The expression of beta-1,3-glucuronyltransferase 3 (glucuronosyltransferase I), involved in the proteoglycan synthesis [46], was upregulated two-fold. GL1-Kruppel family gene [47–49], induced 1.9-fold, is an agonist of BMP-4 and 7, while twisted gastrulation protein, suppressed 1.8-fold, is a BMP competitor [50,51]. The laminin receptor 1, upregulated 1.6-fold, is induced in vitro during chondrogenesis [52].

To validate the changes in gene expression observed in the microarray, ST2 cells were exposed to 1% or 21% oxygen, and the changes in expression of lysyl oxidase, chondroitin-4-sulfonotransferase-2, connective tissue growth factor, and annexin 5 were quantitated using real-time PCR. The expression of each of these mRNAs was significantly upregulated by hypoxia (Fig. 1).

These data suggested that hypoxia induced genotypic changes in ST2 cells consistent with chondrocyte lineage progression. To further investigate this possibility, we examined the pattern of expression of three genes known to be involved in chondrocyte differentiation that were not on the microarray. Real-time PCR was performed on RNA samples isolated from ST2 cells exposed to 1% or 21% oxygen for 0 to 168 h. The expression of Sox9, a key transcription factor required for chondrocyte differ-

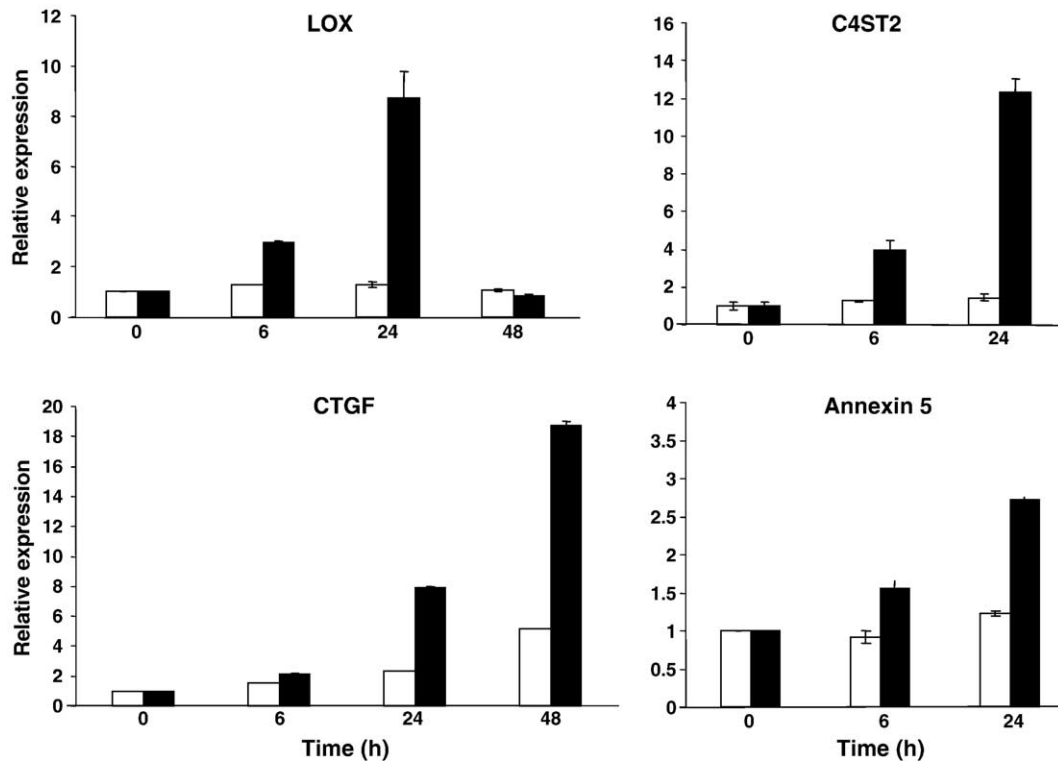


Fig. 1. Effect of hypoxia on the expression of genes associated with chondrocyte differentiation. ST2 cells were cultured, in 21% oxygen, to 70% confluence and then exposed to 1% or 21% oxygen for the times indicated. Real-time PCR was used to confirm the expression of chondrocyte associated genes contained in the microarray. White bars (\pm EM) are from cells exposed to 21% O₂, and black bars (\pm SEM) are from cells exposed to 1% O₂. LOX = lysyl oxidase, C4ST2 = chondroitin-4-sulfonotransferase-2, CTGF = connective tissue growth factor.

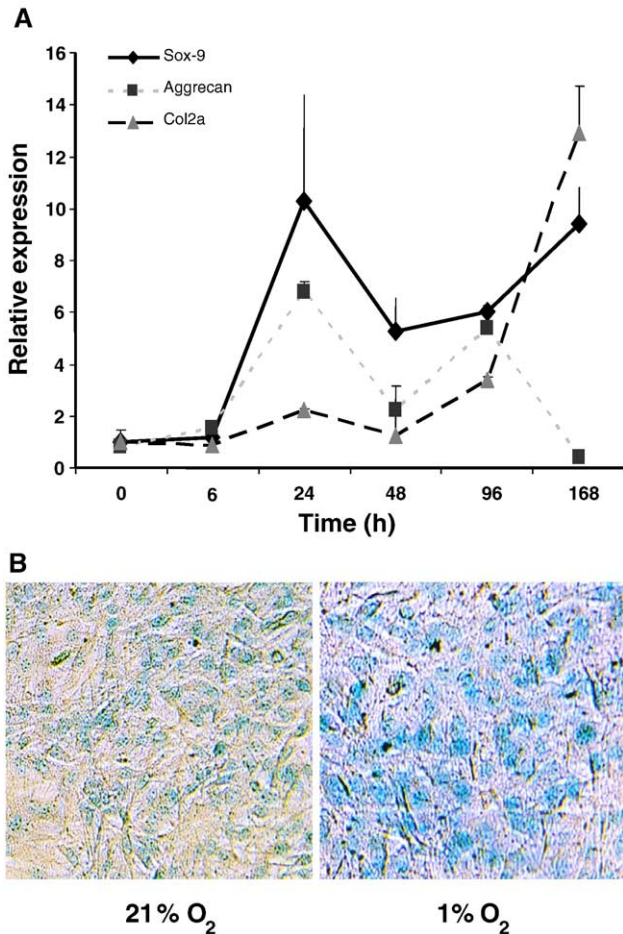


Fig. 2. Genotypic and phenotypic changes in ST2 cells exposed to hypoxia. (A) ST2 cells were cultured at 1% or 21% oxygen for the times indicated. RNA was collected, and real-time PCR was performed to assess the expression of Sox9 (diamonds), Col2a (triangles), and aggrecan (squares). The data are fold increase (\pm SEM) in mRNA expression from cells cultured in 1% O₂ relative to cells cultured in 21% O₂, normalized to time 0. (B) Cells were exposed to 1% or 21% oxygen for 7 days, fixed and stained with alcian blue as described in Materials and methods. Cells cultured in 1% O₂ demonstrated increased staining compared to those cultured in 21% O₂. The results illustrated are representative of 3 independent experiments.

entiation [53], was increased nearly 10-fold at 24 h and remained elevated over the time course of the experiment (Fig. 2). Expression of Col2a, a downstream target of Sox9 [54], was significantly elevated after 96 h and remained elevated at 168 h. The expression of aggrecan, a proteoglycan also known to be under transcriptional control of Sox9 [55], was induced at 24 h (Fig. 2).

To determine whether hypoxia influences the development of the chondrocyte phenotype, we determined the effect of hypoxia on mucopolysaccharides, which are known to accumulate during early stages of chondrocyte differentiation. ST2 cells were cultured in 1% or 21% O₂ for 7 days and then stained for mucopolysaccharides with alcian blue as described in the Materials and methods section. Cells grown in hypoxia demonstrated significantly

greater staining compared to the cells cultured in normoxia (Fig. 2B).

We next sought to determine whether hypoxia could influence the differentiation of another pluripotent mesenchymal-derived cell type. C3H10T1/2 cells are an established mesenchymal stem cell line which can differentiate into muscle, fat, and cartilage cells when treated with azacytidine [56]. In these cells, BMP treatment favors differentiation into chondrocytes and osteoblasts. C3H10T1/2 cells were treated with 400 ng/ml BMP-7 and then cultured in 1% or 21% O₂ for 16 days. Hypoxia increased Col2a and aggrecan mRNA expression (Fig. 3A) and increased the accumulation of mucopolysaccharide as demonstrated by alcian blue staining in comparison to cells treated with BMP-7 maintained under normoxia (Fig. 3B). In a separate experiment, exposure of BMP-treated C3H10T1/2 cells to 1% O₂ for 8 h resulted in a significant induction of Sox9 mRNA (data not shown). Therefore, similar to the results

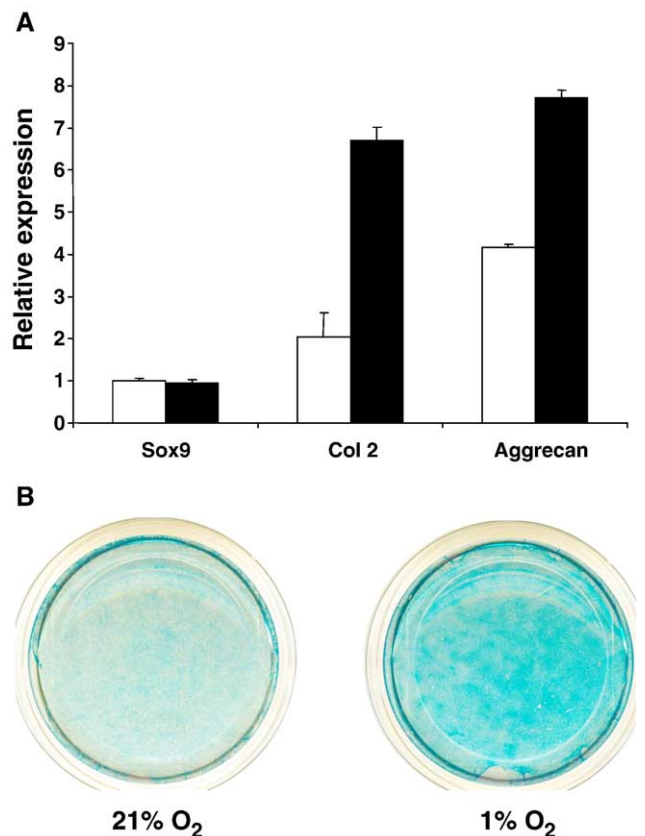


Fig. 3. Hypoxia induces chondrocyte-specific genes and increases mucopolysaccharide accumulation in C3H10T1/2 cells exposed to hypoxia. (A) C3H10T1/2 cells were cultured at 1% (black bars) or 21% (white bars) oxygen for 16 days. RNA was collected, and real-time PCR was performed to assess the expression of Sox9, Col2a, and aggrecan. The data are fold increase (\pm SEM) in mRNA expression. (B) Cells were exposed to 1% or 21% oxygen for 16 days, fixed and stained with alcian blue as described in Materials and methods. Cells cultured in 1% O₂ demonstrated increased staining compared to those cultured in 21% O₂. The results illustrated are representative of 3 independent experiments.

obtained in the ST2 cells, the hypoxia-induced increase in Sox9 mRNA preceded the induction of Col2 and aggrecan.

Hypoxia increases accumulation of HIF-1 α and induces the Sox9 gene promoter

As mentioned above, hypoxia induces the expression of many oxygen-sensitive genes by elevating the level of HIF-1 α which translocates to the nucleus and activates oxygen-sensitive genes by binding with the constitutive HIF-1 β at consensus hypoxia response elements (HREs). To begin to investigate the molecular mechanisms responsible for the induction of the hypoxia-regulated genes, we determined the effect of hypoxia on the accumulation of HIF-1 subunits in nuclear extracts from ST2 cells. Exposure of cells to 1% O₂ resulted in a rapid increase in the levels of HIF-1 α , whereas levels of the constitutive HIF-1 β subunit remained constant as expected (Fig. 4A).

To examine the effect of hypoxia on Sox9 transcription, ST2 cells were transiently transfected with a 6.8 kb Sox9 promoter-luciferase construct [20]. As a control for the assay, ST2 cells were transfected with a -2273 to 51 bp VEGF-luciferase promoter construct known to be transactivated by exposure to hypoxia. Exposure of ST2 cells

to 1% oxygen for 24 h resulted in a 3-fold increase in Sox9 promoter activity and a 4-fold increase in VEGF promoter activity compared to cells exposed to 21% oxygen (Fig. 4B).

As discussed above, HIF-1 α transactivates oxygen-sensitive gene promoters by binding to a consensus hypoxic response element (HRE) comprising the core sequence 5'-GCTGC-3' [57,58]. Inspection of the Sox9 promoter revealed four putative HRE sequences (Fig. 5). To determine the contribution of these sites to promoter induction, a series of 5' deletion mutants were transfected into ST2 cells. The pKp deletion, which removed a cluster of three putative HREs, decreased basal and hypoxia inducible promoter activity by 50%. Further deletion of the proximal HRE severely reduced both basal and hypoxia-induced promoter activity (Fig. 5A). These results suggested that the putative HREs cluster in the Sox9 promoter play a significant role in promoter activation. To examine the individual contribution of these sequences, a series of 3 bp substitutions were introduced to alter the binding site to 5'-GAAAG-3'. Mutation of the HRE on the forward strand (pKp-m1) decreased activity by 50%, while mutations of the putative reverse strand HREs (pKp-m1 and pKp-m2) eliminated promoter activity (Fig. 5B).

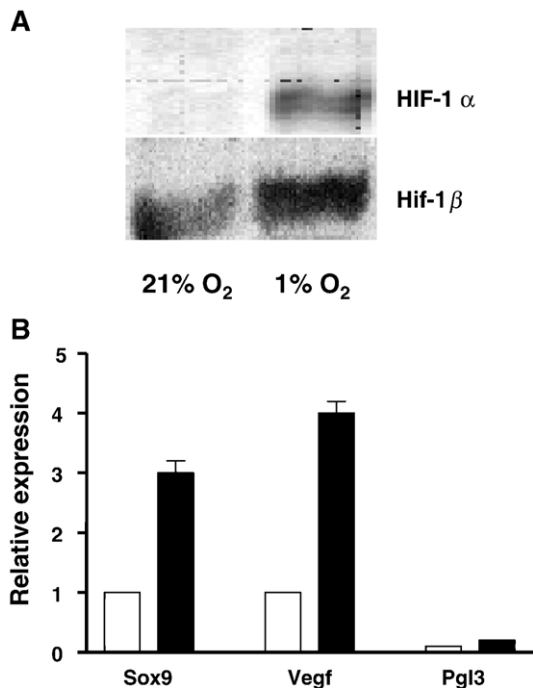


Fig. 4. Hypoxia increases nuclear accumulation of HIF-1 α and activates the Sox9 promoter in ST2 cells. (A) Monolayers were exposed to 1% or 21% O₂ for 4 h. Nuclear extracts were gel separated and immunoblotted with antibodies against HIF-1 α (top) and HIF-1 β (bottom). (B) ST2 cells were transfected with either 1.6 μ g of a pSox9 construct, 1.6 μ g of pVEGF construct, or 1.6 μ g of pGL3 empty plasmid and exposed to 1% or 21% oxygen for 24 h. White bars represent relative luciferase activity (\pm SEM) from cells maintained in 21% O₂, and the black bars represent relative luciferase activity (\pm SEM) from cells exposed to 1% O₂.

Discussion

In this study, we show that hypoxia modulates the fate of mouse ST2 stromal cells by inducing the expression of genes associated with chondrogenesis. The ST2 cells were originally cloned from bone marrow stromal of BC8 mice and have been shown to exhibit properties consistent with pluripotent stromal cells [17]. This cell line has been shown to support both myelopoiesis and B cell lymphopoiesis and to differentiate into bone-forming osteoblasts in response to BMP-2 [59]. The current studies suggest, for the first time, that ST2 cells also have the potential to differentiate along the chondrocyte pathway when exposed to hypoxia.

Gene profiling of RNA from ST2 cells exposed to hypoxia revealed the induction of a number of genes expressed by chondrocytes that were not previously known to be regulated by hypoxia (Table 2). Moreover, both ST2 and C3H10T1/2 cells exposed to hypoxia demonstrated increased accumulation of mucopolysaccharide as indicated by alcian blue staining. Thus, activation of chondrogenic gene pathways appears to promote the development of phenotypic characteristics consistent with chondrocytes. Differentiation of pluripotent stromal cells along a chondrocyte pathway might also be expected to be associated with a decreased expression of genes in the osteoblast or adipocyte program. Indeed, osteocalcin mRNA, a marker of osteoblast differentiation, was significantly decreased in ST2 cells exposed to 1% oxygen (data not shown). Genes associated with adipocyte differentiation, including PPAR γ and RXR α , were unchanged, at least within the 48 h period.

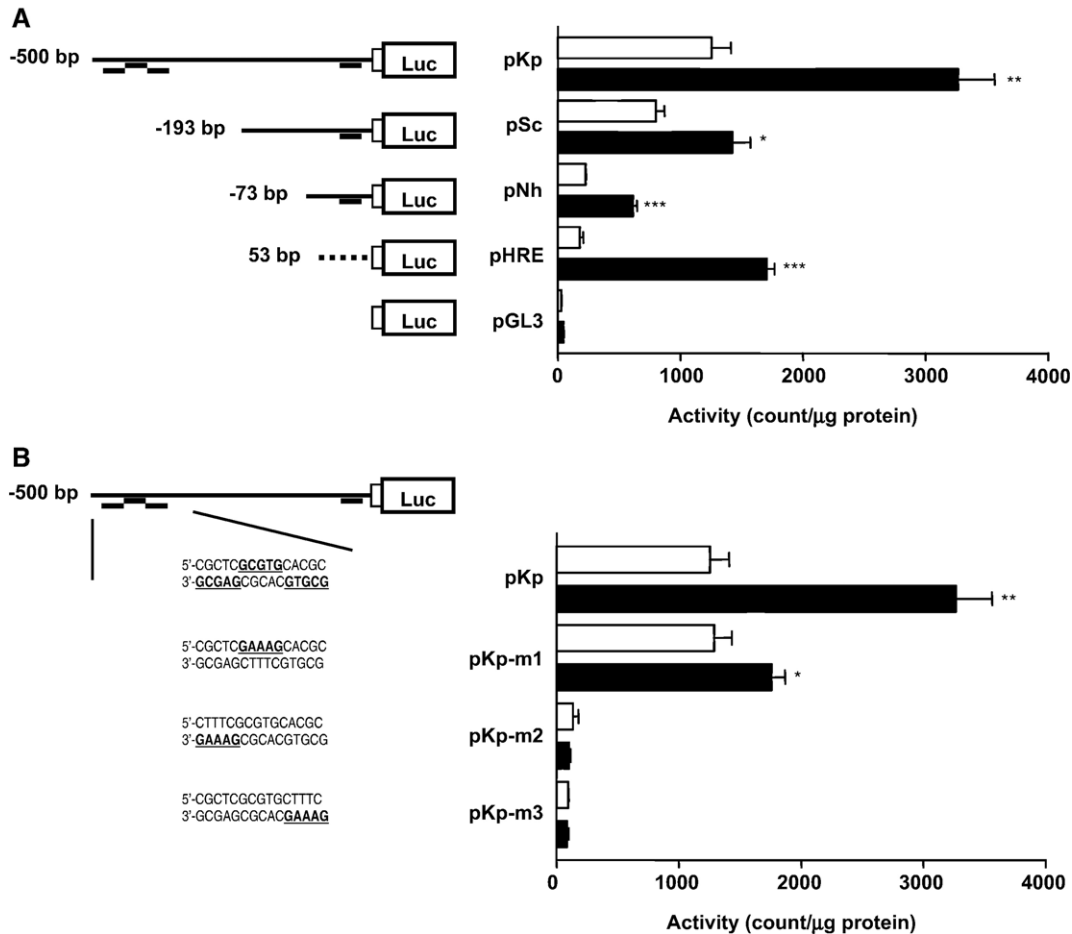


Fig. 5. Functional analysis of the Sox9 promoter in ST2 cells. (A) Restriction analysis of the Sox9 promoter in ST2 cells. Deletion mutants were constructed as described in the Materials and methods and named according to the restriction enzyme used. The 3' end of each construct was fused to the *luc* coding region in a pGL3 basic plasmid. Putative HRE regions are identified with short lines in each construct map. pHRE is a single bona fide HRE site fused to *luc* in pGL3, whereas pGL3 is the empty vector. White bars represent luciferase activity in cells cultured in normoxia, and black bars are from cells cultured in hypoxic conditions. Data presented are mean activity (\pm SEM). (B) Site-directed mutagenesis of the putative HRE sites in the Sox9 promoter. The site of the 3 bp substitutions is illustrated as underlined and bold. Activity of each mutation is illustrated in the graph. White bars represent cells cultured in normoxia, and black bars are from cells cultured in hypoxic conditions. Data presented are mean activity (\pm SEM).

Several previous studies have suggested that oxygen tension influences chondrocyte differentiation. For example, primary cultures of rat mesenchymal stromal cells grew faster when cultured in low oxygen [11], and dedifferentiated articular chondrocytes have been shown to redifferentiate when cultured under hypoxic conditions [60,61]. Furthermore, as stated above, endochondral bone is formed during an avascular period, and chondrocytes in the developing growth plate are hypoxic [62]. In addition, pathophysiologic conditions associated with a low oxygen environment, such as long-bone fracture, result in formation of cartilage. These observations, together with our findings that hypoxia induces the expression of genes expressed by differentiated chondrocytes, suggest that the formation of chondrocytes from bone marrow stromal stem cells is favored in a low oxygen environment.

The finding that Sox9 was acutely upregulated in ST2 cells cultured in low oxygen suggests a potential mechanism for activation of chondrocyte gene programs by hypoxia.

Observations in mice lacking *Sox9* suggest that it mediates early events in the differentiation of mesenchymal progenitor cells into osteochondroprogenitor cells but also functions throughout chondrocyte proliferation and differentiation until the hypertrophic stage [54]. Mutations of *Sox9* in humans result in camptomelic dysplasia, a disorder characterized by abnormal bone formation resulting in short bowed limbs [63,64]. Sox9 promotes chondrocyte differentiation in part by activating chondrocyte-specific enhancer elements in *Col2a* and *aggrecan* [55,65]. This latter finding is supported by our current results which showed that hypoxia induced elevations in Sox9 in association with induction of *Col2a* and *aggrecan* mRNA.

Our findings also suggest that HIF-1 α , a key regulator of the hypoxic response in mammalian tissues, mediates the effect of hypoxia on chondrocyte-specific gene expression. The nuclear accumulation of HIF-1 α protein and the induction of the Sox9 promoter activity both preceded the stimulation of *Col2a* and *aggrecan* mRNA. Moreover,

elimination of a cluster of HIF binding sequences through deletion or mutagenesis profoundly decreased Sox9 promoter activity. The involvement of HIF-1 α in cartilage development is supported by the observations in conditional HIF-1 α knockout mice. In these animals, deletion of HIF-1 α in chondrocytes in the interior of the developing growth plate resulted in premature programmed cell death [62]. Furthermore, chondrocytes lacking HIF-1 α exposed to hypoxia had markedly decreased expression of the HIF-1 α target genes Col2a and aggrecan [66], suggesting the importance of this pathway in chondrocyte lineage progression. Finally, HIF-1 α is also expressed by human chondrocytes, and its expression is upregulated by hypoxia and TNF- α [67].

In summary, we have demonstrated that exposure of mouse stromal ST2 stem cells to low oxygen levels induces genotypic and phenotypic changes consistent with differentiation along a chondrocyte pathway. The effect of hypoxia to promote the progression of the chondrocyte lineage appears to require transcriptional activation by HIF-1 α . These results suggest that the ambient oxygen tension is an important factor in the determination of bone cell fate.

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