Hypoxia Promotes the Differentiated Human Articular Chondrocyte Phenotype through SOX9-dependent and -independent Pathways*

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The chondrocyte is solely responsible for synthesis and maintenance of the resilient articular cartilage matrix that gives this load-bearing tissue its mechanical integrity. When the differentiated cell phenotype is lost, the matrix becomes compromised and cartilage function begins to fail. We have recently shown that hypoxia promotes the differentiated phenotype through hypoxia-inducible factor 2α (HIF-2α)-mediated SOX9 induction of the main matrix genes. However, to date, only a few genes have been shown to be SOX9 targets, while little is known about SOX9-independent regulators. We therefore performed a detailed microarray study to address these issues. Analysis involved 35 arrays on chondrocytes obtained from seven healthy, non-elderly human cartilage samples. Genes were selected that were down-regulated with serial passage in culture (as this causes loss of the differentiated phenotype) and subsequently up-regulated in hypoxia. The importance of key findings was further probed using the technique of RNA interference on these human articular chondrocytes. Our results show that hypoxia has a broader beneficial effect on the chondrocyte phenotype than has been previously described. Of especial note, we report new hypoxia-inducible and SOX9-regulated genes, Gdf10 and Chm-I. In addition, Mip6 and Inhba were induced by hypoxia, predominantly via HIF-2α, but were not regulated by SOX9. Therefore, hypoxia, and more specifically HIF-2α, promotes both SOX9-dependent and -independent factors important for cartilage homeostasis. HIF-2α may therefore represent a new and promising therapeutic target for cartilage repair.

The main function of articular cartilage is to protect the underlying bone by withstanding loading of the joint. Because it is the only cell type in the tissue, the chondrocyte is solely responsible for synthesis and maintenance of the resilient extracellular matrix that gives cartilage its mechanical integrity. The matrix predominantly consists of type II collagen fibers and aggrecan. The former enables resistance to tensional forces, whereas the latter draws water into cartilage and therefore gives the tissue its ability to withstand compressive forces (1, 2). However, when the differentiated cell phenotype is lost, production of these proteins decreases greatly and the matrix becomes compromised, and therefore cartilage function begins to fail. An altered phenotype is found in diseased states such as arthritis, where the cartilage matrix wears away, eventually exposing the underlying bone (3). In addition, current cell-based therapies aimed at cartilage repair such as autologous chondrocyte implantation rely on subculture of chondrocytes ex vivo. However, the differentiated phenotype is also lost in such standard cell culture conditions (4–6).

Cartilage is an avascular tissue and therefore exists in a low oxygen environment (7, 8). We have previously identified hypoxia as a promoter of the differentiated phenotype (9, 10) and shown this occurs through HIF-2α-mediated induction of master chondrocyte regulator, transcription factor SOX9 (11). Sox9 is essential for cartilage development in mice (12), and in humans, heterozygous mutation of the Sox9 gene causes campomelic dysplasia, a severe skeletal disease (13–15). However, to date, only a few genes have been shown to be SOX9 targets (16–18), while little is known about SOX9-independent regulators. Therefore, the aim of the present study was to identify novel regulators of the chondrocyte phenotype. Such findings could be used to improve current cell-based therapies or identify new treatments for restoration and maintenance of cartilage homeostasis in diseased or damaged tissue.

Potential chondrocyte regulators and markers were identified as genes that were down-regulated with serial passage in culture (as this causes loss of the differentiated phenotype) (4–6) and subsequently up-regulated in hypoxia (a condition shown to promote the differentiated phenotype, (9, 10, 19). An extensive microarray analysis was thus performed: 35 arrays from seven individual patient samples, with two time points, and primary chondrocytes from each patient acting as a positive control. The SOX9 dependence of the most promising candidates was subsequently assessed using the technique of RNA interference. In this manner, we identified novel SOX9-dependent and -independent genes relevant to expression of the differentiated human chondrocyte phenotype and hence to cartilage repair.

EXPERIMENTAL PROCEDURES

Human Articular Chondrocyte Isolation and Expansion—Healthy articular cartilage was obtained from the femoral con-
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FIGURE 1. Microarray-based experimental design and analysis to identify genes relevant to the differentiated human chondrocyte phenotype. A, dedifferentiation of chondrocytes was obtained by two rounds of subculture of primary cells (1°). These second passage (P2) cells were then incubated in hypoxia (1% oxygen) to induce redifferentiation or in control normoxia (20% oxygen) for 1 and 4 days. Using genome-wide microarrays, comparisons of gene expression were made between 1° and P2 chondrocytes in normoxia (dedifferentiation) and between P2 chondrocytes in hypoxia and those in normoxia (redifferentiation). B, Venn diagram showing genes significantly down-regulated by passage and subsequently up-regulated by hypoxia at both 1 and 4 days (n = 7, p < 0.01). In addition, a condition of hypoxic induction of ≥1.5-fold was applied, yielding a final group of 101 unique transcripts.

Microarray Sample Preparation—RNA was isolated using the RNeasy kit (Qiagen). Total RNA quality was verified by measuring $A_{260}/A_{280}$ ratio with a spectrophotometer (Nanodrop ND-1000), and an aliquot was analyzed with the Agilent Bioanalyzer 2100 system. 100 ng of total RNA was used for preparing cDNA. The reaction was carried out with T7-oligo(dT) primer and Superscript II (Affymetrix). Second strand cDNA synthesis was done at 16 °C by adding DNA polymerase I and RNase H to the reaction. cDNA was then used for a first in vitro transcription of cRNA using the MEGAScript T7 kit (Ambion). cRNA was treated with the Genechip Sample Cleanup Module (Qiagen), and quality was analyzed with the Agilent Bioanalyzer 2100 system. 600 ng of cRNA was used for a second amplification cycle. For this round, double strand cDNA synthesis was followed by incubation with T4 DNA polymerase (Affymetrix). After cleaning up cDNA with the Genechip Sample Cleanup Module (Qiagen), in vitro transcription of cRNA was done in the presence of biotinylated nucleotides using the Genechip IVT labeling kit (Affymetrix). cRNA was purified again with the Genechip Sample Cleanup Module (Qiagen kit) and quantified (Nanodrop ND-1000). Quality of cRNA was analyzed with the Agilent Bioanalyzer 2100 system. cRNA was further tested by hybridizing to a Test 3.0 Array (Affymetrix). Once samples passed these control measures, 20 μg of biotinylated cRNA were hybridized to Human Genome U133 Plus 2.0 Array (56921 transcripts representing 45500 genes; Affymetrix).

Microarray Data Analysis—Gene expression analysis was performed using Resolver software 7.0 (Rosetta). Data were obtained on human articular chondrocyte cultures from a total of seven patients. The following cultures were prepared for microarray analysis: primary chondrocytes (1°) in 20% oxygen (n = 7) and corresponding second passage (P2) chondrocytes after exposure to 20 and 1% oxygen (n = 7 each). These latter P2 comparisons were done after both 1 and 4 days of treatment, giving a total data set of (5 × 7) 35 arrays.

Real-time PCR—This was performed on a real-time PCR Corbett Research thermocycler as previously described (11). In all cases, pre-developed primer/probe sets were obtained from Applied Biosystems. cDNA was generated from 1 μg of RNA (Qiagen) using the Promega kit with random primers (Promega). Expression was normalized using ribosomal protein (RPLP0), as previous experiments had shown this gene was not regulated by hypoxia.

siRNA Transfection—Human articular chondrocytes were transfected as previously described (11). Briefly, $5 \times 10^6$ cells were seeded in a 3.5-cm dish. siRNA was transfected at a final concentration of 10 nm using Lipofectamine 2000 (Invitrogen) for 4 h in serum-free OptiMEM I. siRNAs against HIF-1α, HIF-2α, and SOX9 were used (11). siRNA against luciferase (Dharmacon) was transfected as a non-targeting control. Efficiency of transfection was also assessed using a fluorescently labeled siRNA (siGlo) from Dharmacon. Four to six hours after trans-
fection, medium was changed with pre-equilibrated Dulbecco's modified Eagle's medium (in 20 or 1% oxygen) containing 10% fetal calf serum and cells were incubated in 20 or 1% oxygen for 3 days.

Protein Extraction and Western Blotting—Cells were lysed in urea sample buffer (8 M urea, 10% glycerol, 1% SDS, 5 mM dithiothreitol, 10 mM Tris-HCl) for HIF analysis or radioimmune precipitation assay buffer. A protease inhibitor mixture (Sigma) was added just prior to cell lysis. Protein (20 μg) was separated by SDS-PAGE, transferred onto polyvinylidene difluoride membranes for Western blotting, and finally visualized using the ECL method. Primary antibodies

| Genes down-regulated with passage and subsequently up-regulated in hypoxia in human articular chondrocytes |
| 101 genes were identified whose expression was down-regulated in twice-passaged chondrocytes (P2 chondro) compared to primary cells (1° chondro) and subsequently up-regulated in hypoxia after 1 and 4 days of treatment (n = 7, p < 0.01, hypoxic fold induction ≥ 1.5). Genes have been classified according to their function and/or cellular localization. Microarray expression intensities (AU, arbitrary units) for 1° cells are presented in the left column. Real-time PCR was performed for 23 genes by way of validation of the microarray data. The two righthand columns show real-time PCR data (obtained from cells from one patient), while the corresponding microarray data represent the average from n = 7 patients.

**Table 1**

| Signaling | Matrix Genes |
|-----------|--------------|
| 43 | WW52 |
| 37 | TME45A |
| 4 | EP5L2 |
| 10 | 15K |

| Growth factors and binding proteins |
|-----------------------------------|
| 18 | 12 | 6 |

| Membrane proteins |
|--------------------|
| 2 | 3 | 5 |

| Metabolism |
|------------|
| 8 | 10 | 12 |

| Cellular integrity |
|--------------------|
| 17 | 10 | 8 | 7 | 5 |

Oxothiol, 10 mM Tris-HCl) for HIF analysis or otherwise in radioimmune precipitation assay buffer. A protease inhibitor mixture (Sigma) was added just prior to cell lysis. Protein (20 μg) was separated by SDS-PAGE, transferred onto polyvinylidene difluoride membranes for Western blotting, and finally visualized using the ECL method. Primary antibodies
used were rabbit anti-SOX9 (AB5535, 1:1000; Chemicon), mouse anti-HIF-1α (clone 54, 1:250; BD Transduction Laboratories), mouse anti-HIF-2α (Sc-13596, 1:250; Santa Cruz Biotechnology), mouse anti-α-tubulin monoclonal antibody (Sigma). MIG6 antibody was a kind gift from Dr. Ingvar Ferby.

Statistical Analysis—Unless otherwise stated, data were compared using one-way analysis of variance with Bonferroni’s post test using Prism 4 software (GraphPad). Results are mean ± S.E. from four to seven independent experiments (i.e. using chondrocytes from four to seven individual patients). p < 0.05 was considered statistically significant.

RESULTS

The Main Cartilage Matrix Genes Are Hypoxia-inducible—An extensive microarray analysis was performed using 35 genome-wide arrays on human articular chondrocytes obtained from seven patients (showing no signs of cartilage disease). Genes potentially relevant to the differentiated phenotype were identified as those whose expression was decreased with serial passage of chondrocytes in normoxia (dedifferentiation) and subsequently up-regulated in hypoxia (redifferentiation) (Fig. 1A). A final list of 101 transcripts was obtained for which their expression met these criteria at both 1- and 4-day time points (n = 7, p < 0.01) (Fig. 1B). A threshold for hypoxic induction ≥1.5-fold was also applied. Results have been classified according to known function in Table 1, which also shows validation of microarray data by real-time PCR (for 23 genes). All of the established cartilage extracellular matrix genes (aggrecan, Col2a1, Col9a1, Col11a2), in addition to master chondrocyte regulator transcription factor Sox9 were identified as hypoxia-inducible genes (Table 1). In addition, the extracellular matrix protein Prelp was both highly expressed in pri-
primary human chondrocytes and hypoxia-inducible (Fig. 2D). Of the matrix proteins, higher expression in primary chondrocytes was only observed for aggrecan and Col2a1 (Fig. 2, A and B, respectively, and Table 1).

Applying an unsupervised genome-wide hierarchical cluster analysis, the cartilage-associated genes Hapln1 (link protein), Fgfr3, Ctgf, and the recently discovered cartilage collagen Col27a1 (20) were shown to cluster tightly with the previously identified aggrecan, Col2a1, and Col11a2 (Fig. 3). Because this analysis was performed on the whole genome for the complete data set of 35 arrays, the cluster containing these genes indicates a very similar pattern of expression through the dedifferentiation/redifferentiation process, albeit with some variation between patient samples.

Hypoxia-inducible Genes Gdf10 and Chm-I Are SOX9-dependent—Chm-I and Gdf10 were identified as hypoxia-inducible genes relevant to the differentiated chondrocyte phenotype (Fig. 2, G and H, respectively). To more directly assess the potential importance of these genes to cartilage function, we investigated their dependence on the master cartilage transcription factor SOX9 by depleting the latter using RNA interference. Fig. 4A shows that virtually all the cells were successfully transfected using Lipofectamine to deliver the siRNA oligonucleotides, with no detrimental effects on cell viability. SOX9 depletion was comprehensive as shown by Western blotting (Fig. 4B). The expression of both Chm-I and Gdf10 was significantly reduced in both 20 and 1% oxygen in SOX9-depleted cells. Specifically, hypoxic induction of Chm-I was completely abolished by depletion of SOX9 (Fig. 4C), while that of Gdf10 was greatly reduced (Fig. 4D).

INHBA and MIG6 Are Potential Chondrocyte Regulators Whose Expression Is Independent of SOX9—The highly expressed Mig6 (Fig. 2C) and InhbA (Fig. 2E) were both identified through microarray analysis as potential regulators of the chondrocyte phenotype. In addition, InhbA clustered with Col2a1 and aggrecan, among other cartilage-specific proteins,
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in a hierarchical cluster analysis (Fig. 3). Neither Mig6 (Fig. 5, C and D) nor InhibA (Fig. 5E) was found to be SOX9-dependent. However, when HIF-1α and HIF-2α were depleted in human chondrocytes (Fig. 5, A and B, respectively), we showed that Mig6 hypoxic induction was specifically dependent on HIF-2α (Fig. 5, C and D), whereas InhibA was both HIF-1α- and 2α-dependent (Fig. 5E).

DISCUSSION

The aim of this study was to identify novel regulators and markers of the differentiated human chondrocyte phenotype. This is important because repair of damaged and diseased cartilage relies on appropriate matrix synthesis from the resident chondrocytes. Using a microarray-based approach we identified a tightly regulated group of potentially important chondrocyte genes, whose regulation was further probed using the technique of RNA interference. We thus identified matrix protein Chm-I and growth factor Gdf10 as new SOX9-regulated genes and Mig6 and InhibA as hypoxia-inducible (predomi-}

nantly through HIF-2α), but SOX9-independent, genes defining the differentiated human chondrocyte phenotype.

This study was performed using chondrocytes from healthy human cartilage, i.e. non-diseased and non-elderly tissue (mean age of 22 years). Typically, human cartilage research is done on the only tissue readily available: elderly, arthritic cartilage obtained following joint replacement. However, our samples were obtained from amputations due to sarcomas that did not involve the joint space. Thus this tissue represents a rarely obtained and highly therapeutically relevant research tool. With normal cartilage obtained from a total of seven different patients, we identified potential chondrocyte regulators by analyzing their changing expression in three different conditions: primary chondrocytes indicating the normal, differentiated phenotype; dedifferentiated chondrocytes obtained through serial subculture; and redifferentiating chondrocytes (by exposure of dedifferentiated cells to hypoxia). It has long been known that serial subculture of chondrocytes leads to loss of phenotype (4–6). In addition, recent evidence has shown that hypoxia promotes the differentiated phenotype (9, 10, 19) and that the master chondrocyte regulator SOX9 is instrumental in this (11). We therefore applied this double-comparison analysis (genes down-regulated with passage and subsequently up-regulated in hypoxia) to the present study to ensure that genes thus identified were not simply hypoxia-inducible but were also potentially relevant to the differentiated cell phenotype. This is in contrast to the single-comparison analyses that are normally made when adopting a microarray approach, e.g. diseased versus“normal” (21, 22) or differentiated versus dedifferentiated chondrocytes (23). The inclusion of two time points (1 and 4 days) and the use of cells from seven different patients further strengthened the analysis and gave a comprehensive data set based on 35 genome-wide arrays. The established and cartilage-specific matrix genes were thus identified as hypoxia-inducible (i.e. aggrecan, Colla1, Col9a1, Col11a2), in addition to transcription factor SOX9, which controls expression of these genes (11, 16, 18). The cluster analysis did reveal variations in the degree of this response between samples, which most likely reflects the fact that each sample represents cells from each of seven individual patients rather than, for example, a


gene expression data, mRNA was isolated 3 days after transfection of SOX9-targeting siRNA (siSOX9) and transcripts were analyzed by real-time PCR, normalizing expression levels to endogenous control RPLP0. Values are means ± S.E. *, p < 0.05; **, p < 0.01 (paired t test).

FIGURE 4. New hypoxia-inducible, SOX9-dependent genes identified in human articular chondrocytes. In all experiments, data are shown from second passage human articular chondrocytes. Luciferase-targeting siRNA (siLuc) were transfected as a control, and all siRNA were used at a concentration of 10 nm. A, use of fluorescently labeled siRNA (siGlo) (Dharmacon) demonstrated that virtually all of the cells were successfully transfected with no adverse effect on cell viability. B, SOX9 was comprehensively depleted as determined by Western blotting. C, anti-angiogenic factor Chm-I was strongly SOX9-dependent in both hypoxia and normoxia. Data are from cultures from n = six patients. D, growth factor Gdf10 was also highly hypoxia-inducible and SOX9-dependent (n = 4). For gene expression data, mRNA was isolated 3 days after transfection of SOX9-targeting siRNA (siSOX9) and transcripts were analyzed by real-time PCR, normalizing expression levels to endogenous control RPLP0. Values are means ± S.E. *, p < 0.05; **, p < 0.01 (paired t test).
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![Graphs and images]

**FIGURE 5.** InhBA and MIG6 are regulated by hypoxia through the HIF pathway, but independently of SOX9. SOX9, HIF-1α, HIF-2α, and luciferase (Luc; control) were depleted by transfecting cells with gene-specific siRNA. All data shown are from second passage human articular chondrocytes. In all experiments, cultures were harvested 3 days after exposure to hypoxia. Western blot showing successful depletion of HIF-1α (A) and HIF-2α (B) in hypoxic conditions (1% oxygen). SOX9 depletion is shown in Fig. 4B. Western blot showing MIG6 protein was strongly hypoxia-inducible and this induction was greatly reduced specifically by depletion of HIF-2α, but not HIF-1α or SOX9. **C**. D, hypoxic induction of MIG6 mRNA was similarly specifically HIF-2α-dependent. Data from n = four cultures (cells from four patients). **D**, hypoxic induction of InhBA was HIF-1α- and HIF-2α-dependent, but SOX9-independent (n = 4). For gene expression data, transcripts were analyzed by real-time PCR, normalizing expression levels to endogenous control RPLP0. Values are means ± S.E. **E**, p < 0.001 versus siLuc in 1% oxygen.

single cell line with replicates. Therefore these findings confirmed the validity of our approach of using hypoxia (1% oxygen) to restore the differentiated human articular chondrocyte phenotype. Because cartilage is avascular, the chondrocytes are exposed to low oxygen levels in vivo. A gradation of oxygen tension from 2 to 7% has been measured across the epiphyseal plate in rats and rabbits (7), whereas lower tensions would be expected in (thicker) human cartilage. Our results highlight the importance of studying chondrocyte function in such physiological levels of oxygen (i.e., hypoxia) compared with standard in vitro conditions (20% oxygen).

Using RNA interference, we provide the first evidence that Chm-1 and Gdf10 are SOX9-regulated genes. In fact, their hypoxic induction was abolished by SOX9 depletion. Hypoxic induction of matrix protein Prelp was also reduced in SOX9-depleted cells, although not to a statistically significantly degree (data not shown). ChM-1 is a relatively cartilage-specific secreted protein that has been shown to have anti-angiogenic properties (24). Therefore, our current finding that it is hypoxia-inducible and that this is highly SOX9-dependent offers an entirely plausible mechanism for its regulation because the permanent articular cartilage, with its primary function of withstanding high mechanical loads, cannot afford a delicate blood supply and therefore must constantly inhibit vascular invasion throughout life.

Although various growth factors (including transforming growth factor β1 (TGF-β1), TGF-β3, bone morphogenetic protein, and fibroblast growth factor family members) have been used in different human chondrocytes studies (25–27), there is little evidence that these molecules are actually endogenously produced in significant amounts by adult human articular cartilage. In the current study we identified two TGF-β superfamily members, GDF10 and INHBA. The latter dimerizes to form activin-A. In a recent proteomic study, colleagues in our institute have identified activin-A as a major secreted factor from human cartilage explants (28). They further demonstrated that activin-A induces tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) in human chondrocytes. Activin-A may therefore act as an anti-catabolic factor in cartilage, because TIMP-1 inhibits the matrix-degrading enzymes matrix metalloproteinases (MMPs). Indeed, Chang et al. (29) recently reported that activin-A inhibits MMP-3 in human chondrosarcoma cells and conclude that activin-A may have therapeutic potential as a chondroprotective agent. The present study provides the first evidence that this endogenously produced factor is hypoxia-inducible and furthermore that this induction is independent of SOX9.

MIG6 was also identified as a potential chondrocyte regulator and was found to be very highly expressed in normal human chondrocytes. Although its mechanism of action in cartilage is not well defined, its importance to maintenance of the tissue is shown by the fact that deletion of the gene in mice leads to early onset joint disease with marked cartilage...
Hypoxia promotes the differentiated human articular chondrocyte phenotype through SOX9-dependent and -independent pathways. In addition to the cartilage-specific collagens and aggrecan, we identified anti-angiogenic factor Chm-I and growth factor Gdf10 as new SOX9-dependent genes, whereas Inhba and Mig6 are regulated via the HIF pathway (predominantly HIF-2α) independently of SOX9.

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