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Hypoxia Modulates the Phenotype of Osteoblasts Isolated From Knee Osteoarthritis Patients, Leading to Undermineralized Bone Nodule Formation

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Objective. **To investigate the role of hypoxia in the pathology of osteoarthritic (OA) bone by exploring its effect on the phenotype of isolated primary osteoblasts from patients with knee OA.**

Methods. **OA bone samples were collected at the time of elective joint replacement surgery for knee or hip OA. Normal bone samples were collected postmortem from cadaver donors. Primary osteoblasts were isolated from knee OA bone chips and cultured under normoxic or hypoxic (2% O_2) conditions. Alkaline phosphatase activity was quantified using an enzymatic assay, and osteopontin and prostaglandin E_2 (PGE_2) production was assayed by enzyme-linked immunosorbent assay. Total RNA was extracted from bone and osteoblasts, and gene expression was profiled by quantitative reverse transcription–polymerase chain reaction.**

Results. **Human OA bone tissue sections stained positively for carbonic anhydrase IX, a biomarker of hypoxia, and exhibited differential expression of genes that mediate the vasculature and blood coagulation as compared to those found in normal bone. Culture of primary osteoblasts isolated from knee OA bone under hypoxic conditions profoundly affected the osteoblast phenotype, including the expression of genes that mediate bone matrix, bone remodeling, and bone vasculature. Hypoxia also increased the expression of cyclooxygenase 2 and the production of PGE_2 by OA osteoblasts. Osteoblast expression of type II collagen α1 chain, angiopoietin-like 4, and insulin-like growth factor binding protein 1 was shown to be mediated by hypoxia-inducible factor 1α. Chronic hypoxia reduced osteoblast-mineralized bone nodule formation.**

Conclusion. **These findings demonstrate that hypoxia can induce pathologic changes in osteoblast functionality consistent with an OA phenotype, providing evidence that hypoxia is a key driver of OA pathology.**

Although osteoarthritis (OA) has historically been considered a disease of the cartilage, recent evidence from animal models and clinical studies suggest that pathologic remodeling of the subchondral bone (1–5) precedes and mediates the changes observed in the cartilage (5–11). Therefore, understanding the key molecular pathways that mediate pathologic bone remodeling in OA is critical in attempting to identify targets for the development of new therapeutics that can modify disease progression.

Importantly, knee and hip OA are both associated with vascular-related comorbidities (12–16), suggesting a role of dysfunctional vasculature in OA pathology. It is known that disruption of the vascular supply and accompanying hypoxia are associated with modulated bone formation during development and fracture repair (17), and there is now evidence that vascular dysfunction may also play a role in mediating subchondral bone remodeling in OA (13,18).

Subchondral regions of the long bones are highly vascularized and likely provide important nutrition to the growth plate and articular cartilage, suggesting that

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compromised bone vasculature could affect both bone and cartilage health. The subchondral bone in hip and knee OA exhibits evidence of vascular dysfunction, with vascular occlusion and reduced venous outflow (19,20), which may result in local hypoxia (21). Furthermore, bone ischemia has been linked to bone marrow lesions, which are one of the morphologic hallmarks of OA bone and are strong predictors of progressive cartilage degeneration and pain. Recent analysis of bone marrow lesions from patients with knee OA showed that these lesions consisted of highly undermineralized sclerotic bone, with histopathologic features of secondary modeling of vessels, evidence of fibrosis, and multiple thrombus inclusions, consistent with a localized oxygen deficit (18). Therefore, a hypoxic microenvironment could be responsible for driving sclerotic bone formation in OA.

Few studies have reported the role of hypoxia in mediating OA bone pathology. Studies in osteoblast cells lines and in murine osteoblasts suggest that hypoxia can mediate osteoblast activity (22,23), but to our knowledge, the effect of hypoxia on primary osteoblasts isolated from patients with OA has not been reported. As such, this potentially key driver of OA bone pathology is currently poorly understood.

In this study, we first examined subchondral bone obtained from patients with knee and hip OA for evidence of biomarkers of hypoxia and the expression of genes that mediate vasculature and blood coagulation. We then isolated primary osteoblasts from the tibial plateaus of patients with knee OA and examined the effect of hypoxia on osteoblast activity and osteoblast phenotype by measuring the expression of genes implicated in bone remodeling, osteoblast/osteoclast coupling, inflammation, and bone vasculature. In addition, we examined the effect of chronic hypoxia on the ability of primary knee OA osteoblasts to form mineralized bone nodules.

MATERIALS AND METHODS

Tissues. Human OA subchondral joint tissue was obtained at the time of total knee and hip joint replacement operations. Normal bone was obtained postmortem from cadaver donors with no history of joint pain (King's Mill Hospital, Sutton-in-Ashfield, UK). Following surgical removal, OA and normal tissues were left to "rest" for 24 hours in Dulbecco's modified Eagle's medium (DMEM) prior to processing. The cartilage was then cut away from the tibial plateau, and the bone was snap-frozen in liquid nitrogen. Processing times were recorded to ensure that comparisons could be made. Tissue samples from cadaver donors that had evidence of OA on histologic examination were excluded from the study.

For immunohistochemical studies, preliminary experiments were conducted to determine the effect of time delay on the outcome of immunohistochemical analysis, ensuring that samples were processed within an acceptable time frame. Samples were fixed in 10% buffered formalin and decalcified using an EDTA-based protocol. Tissues were dissected to produce samples containing cartilage with underlying subchondral bone, and were then processed and embedded in paraffin wax.

Full ethical consent was obtained from all tissue donors or their families.

Immunohistochemical staining of human OA bone sections. Immunohistochemical staining for the hypoxia biomarker carbonic anhydrase IX (CAIX) was performed on 4-μm–thick sections obtained from OA and normal control tissues. Antigen retrieval was performed by incubation overnight at 60°C in 0.2*M* boric acid. CAIX was detected by incubation for 60 minutes at room temperature with 1 μg/ml of rabbit anti-human CAIX antibody (ab15086; Abcam). Detection was achieved using a biotinylated swine anti-rabbit antibody (E0353; Dako), and StreptABComplex (K0377; Dako). Visualization was performed using 3,3′-diaminobenzidine (Dako) with hematoxylin counterstain. The isotype control we used was human adsorbed rabbit IgG (PRABP01; Serotec) diluted to the same concentration as the primary antibody.

Gene expression analysis of bone and primary human osteoblasts. TRIzol (Invitrogen) was used to extract total RNA from the medial and lateral tibial plateaus of the knees of age- and sex-matched OA and normal donors (n = 10 per group). RNA quality was assessed with an Agilent Bioanalyzer, and all samples had RNA integrity numbers that were >8.0. Gene expression analysis in 10 μg of total RNA was performed using microarray gene chips (Human Genome U133 Plus 2.0 Arrays; Affymetrix). Arrays were hybridized for 16 hours at 45°C, washed, and stained with biotinylated antistreptavidin antibodies and with streptavidin–phycoerythrin (Life Technologies). Images of the resulting arrays were scanned, and quality control was determined using the following criteria. All scaling factors obtained from each sample were confirmed to fall within 3-fold of each other to enable cross-chip comparisons. The percentage of present calls was between 45% and 55%. The 3′:5′ ratio of GAPDH and actin was ≤3, which confirmed that a good length of labeled transcript was generated. All spike-in hybrid controls were called as present.

All data that passed these quality control criteria were then analyzed using a Robust Multiarray Average algorithm. Principle components analysis was performed, which showed good separation of OA and normal control RNA from both the lateral and medial tibia plateau compartments (data available upon request from the corresponding author). To identify differentially expressed genes, analysis of variance (ANOVA) was performed, and a false discovery rate (FDR) was calculated for the entire study. The differentially expressed genes were ranked according to the FDR, and *P* values and fold-changes were determined (data available upon request from the corresponding author).

Total RNA was extracted from primary osteoblasts using RNeasy columns (Qiagen), and gene expression in osteoblasts was quantified by quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis, using Microfluidic TaqMan (Applied Biosystems). C_t values were normalized to 18S RNA, and the comparative C_t method ($\Delta\Delta C_t$) was used for the relative quantification of gene expression.

Ingenuity Pathway Analysis (IPA). The Affymetrix gene expression data were uploaded to the IPA application (www.ingenuity.com), and a core functional analysis was per-

formed to identify canonical pathways that were most significantly associated with the differentially expressed genes (using a cutoff of >1.5-fold differentiation and $P < 0.05$). The significance of the association of a given canonical pathway and the differentially expressed gene targets was measured in two ways. First, the ratio of the number of genes in the network that mapped to the canonical pathway divided by the total number of genes that mapped to the canonical pathway was calculated. Second, Fisher's exact test was used to calculate the *P* value for the association between the genes in the network and the canonical pathway. We then analyzed the overlay of the differentially expressed genes identified by the Affymetrix analysis with the specific gene networks.

Preparation of primary osteoblasts. To isolate primary osteoblasts, cartilage was removed from the femoral condyles and tibial plateaus obtained from the patients with knee OA. The revealed trabecular bone was cut into small pieces measuring 2 mm^3 and washed in growth medium (DMEM supplemented with 10% fetal calf serum, 200 units/ml of penicillin, 100 μg/ml of streptomycin, 2 m*M* L-glutamine, 1% nonessential amino acids [Invitrogen] and 2.5 μg/ml of amphotericin, 2 m*M* β-glycerophosphate, 50 μg/ml of ascorbic acid, and 10 n*M* dexamethasone). Bone explants were cultured in 125-cm^2 cell culture flasks, and the medium was replaced every 3–4 days until cells migrated out of the explants. Confluency was reached in 21 days, at which time the cells were used (passage 0) or were split and grown to confluence again (passage 1). Isolated primary osteoblasts exhibited positive expression of RUNX-2, expressed osteopontin protein and alkaline phosphatase activity, and were also capable of mineralization and forming bone nodules when allowed to become highly confluent. On passaging the osteoblasts, there was evidence of changing phenotype, with a significant reduction in alkaline phosphatase activity, loss of osteopontin protein expression, and loss of RUNX2 expression at passage 1 (data not shown). Therefore, in all experiments, primary osteoblasts were used without additional passaging to avoid any potential phenotypic drift.

At the end of the culture period, osteoblasts in monolayer were scraped into culture medium and centrifuged. Cell pellets were resuspended in either radioimmunoprecipitation assay (RIPA) buffer (50 m*M* Tris HCl, 150 m*M* NaCl, 1 m*M* EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], protease, and phosphatase inhibitor cocktails) for protein isolation or RLT buffer (Qiagen) for RNA isolation. Cell culture supernatants were also collected. Samples were stored at −80°C prior to analysis.

To stimulate the effect of hypoxia on bone, osteoblasts were cultured under 2% oxygen conditions and in preconditioned hypoxic growth medium.

Alkaline phosphatase activity. Total protein was quantified using the bicinchoninic acid protein assay reagent (Pierce). Alkaline phosphatase activity was tested with a biochemical assay involving the conversion of *p*-nitrophenyl phosphate into *p*-nitrophenol. An aliquot of the cell suspension in RIPA buffer was assayed for alkaline phosphatase activity using *p*-nitrophenyl phosphate solution (Sigma). Enzyme activity was expressed as units per milligram of protein.

Mineralization of primary human osteoblasts. Osteoblast mineralization was assessed by alizarin red S staining (Sigma) of cells grown on 24-well plates. Cells were fixed with 10% formalin and stained with 40 m*M* alizarin red S (pH 4.1). To quantify the degree of staining in each of the cultures, the cultures were destained for 10 minutes at room temperature in 100 m*M* cetylpyridinium chloride. Aliquots of each sample were transferred in triplicate to a 96-well plate, and the absorbance was measured at a wavelength of 570 nm.

Detection of hypoxia-inducible factor 1α (HIF-1α) protein. Following incubation at 2% O_2, osteoblasts were removed from the incubator and immediately lysed in RIPA buffer containing a protease inhibitor cocktail. Whole cell lysates were then separated by SDS–polyacrylamide gel electro-

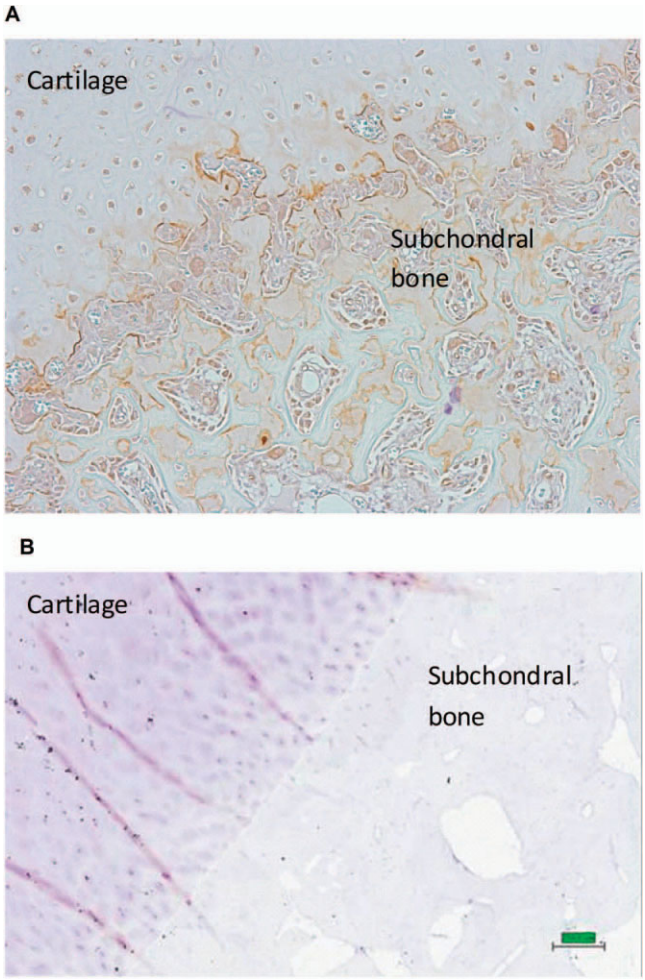

Figure 1. Immunohistochemical detection of carbonic anhydrase IX (CAIX) in osteoarthritic (OA) bone. Samples of human OA femoral heads containing cartilage and underlying subchondral and trabecular bone were stained for the presence of CAIX protein. CAIX was detected in chondrocytes, osteoblasts, osteoclasts, and endothelial cells, as well as within calcified cartilage matrix, in OA bone and was associated with regions undergoing active remodeling. **A,** CAIX staining of an OA bone sample appears to be increased in regions associated with active bone formation, as indicated by high numbers of activated osteoblasts. **B,** CAIX staining of a normal bone sample was limited to the periarticular connective tissue, where it was seen in the medial layer, with some blood vessels. Staining was negative within the subchondral region. IgG control also showed negative staining within the subchondral region (results not shown). Bar = 250 μm. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.38403/abstract.

phoresis, blotted onto an enhanced chemiluminescence (ECL) membrane (GE Healthcare), probed with an HIF-1α polyclonal antibody at 1:500 dilution (Cell Signaling Technology), and visualized using ECL Plus detection (GE Healthcare).

Determination of osteopontin protein and prostaglandin E_2 (PGE_2) levels by enzyme-linked immunosorbent assay (ELISA). Conditioned medium from cultured primary osteoblasts were assayed for PGE_2 using a commercially available ELISA kit (KGE004; R&D Systems) and following the manufacturer's instructions. For the determination of osteopontin protein, we used an in-house–developed ELISA. Briefly, 384-well high-binding polystyrene plates (Costar) were coated with capture antibody (R&D Systems) in phosphate buffered saline (PBS) and left overnight at 4°C. Following removal of the coating antibody, the plates were washed 3 times with wash buffer (PBS containing 0.05% polysorbate), and the wells were blocked for 1–2 hours at room temperature with 100 μl/well of PBS containing 1% bovine serum albumin. The plates were then washed as above, and 40 μl of sample or standard was added to each well. Biotinylated anti-osteopontin (20 μl/well; R&D Systems) was added immediately, and the plate was left for 2 hours before being aspirated. The plate was then washed before the addition of horseradish peroxidase substrate (diluted 1:2,500 in PBS) for 1 hour. All plates were developed with the addition of 40 μl/well of 3,3′,5,5′-tetramethylbenzidine (TMB) for 20 minutes at room temperature in the dark. The reaction was stopped with the addition of H_2SO_4 (2*M*), and the absorbance was read at 450 nm (PheraStar plate reader).

Statistical analysis. All data are reported as the mean ± SEM. Comparisons were performed using ANOVA, with post hoc tests (least significant difference) used to determine significance. *P* values less than 0.05 were considered significant.

RESULTS

Elevated expression of a hypoxia biomarker in OA bone and differential expression of genes that mediate the vasculature and blood coagulation. Immunohistochemical staining of femoral head bone tissue sections for CAIX, a known biomarker of hypoxia, showed positive staining in OA subchondral bone sections. CAIX was detected in chondrocytes, osteoblasts, osteoclasts, and endothelial cells, as well as within calcified cartilage matrix in OA bone (Figure 1A). CAIX staining also appeared to be particularly pronounced in regions associated with new bone formation, as indicated by the high numbers of activated osteoblasts. Samples of normal femoral heads showed negative staining in the subchondral bone region, although there was staining for CAIX within the medial layer of some blood vessels in the periarticular connective tissue (Figure 1B).

Analysis of gene expression in OA and normal subchondral tibial plateau bone revealed evidence of altered expression of several genes in OA bone that are known mediators of the vasculature and of blood coagulation, with similar findings in tissues from both the medial and lateral tibial plateaus (Table 1). The most

Table 1. Differential expression of genes that mediate the vasculature and blood coagulation in OA tibial plateaus*

Gene	Gene symbol	Fold change		Function
		MTP	LTP	
Angiogenesis and vascular modulation				
Adrenomedullin	ADM	−18.0	−23.0	Vasodilation; functions through RAMP receptors
Angiopoietin-like 4	ANGPTL4	−3.0	−3.0	Proangiogenic; apoptosis survival factor for vascular endothelial cells
Collagen triple-helix repeat–containing 1	CTHRC1	4.6	4.5	Vascular repair; induced by fibroblasts and modulates TGFβ signaling
EGF-like repeats and discoidin I–like domains 3	EDIL3	2.2	2.3	Proangiogenic; expressed by endothelial cells in response to VEGF and proinflammatory cytokines IL-1β and TNFα
Osteoglycin	OGN	2.7	3.2	Vascular repair; increased during vascular injury
Receptor (G protein–coupled) activity–modifying protein 1	RAMP1	1.5	1.7	Vasodilation; interacts with adrenomedullin
Vascular endothelial growth factor A	VEGFA	−2.1	−2.1	Proangiogenic; promotes endothelial cell growth and migration
Coagulation				
CD84 antigen (leukocyte antigen)	CD84	3.1	3.1	Platelet aggregation; homophilic adhesion molecule promotes thrombosis
Plasminogen activator inhibitor 1	SERPINE1	−2.0	−2.7	Procoagulant; inhibits plasmin activation and promotes fibrinolysis
Tissue factor pathway inhibitor	TFPI	−3.6	−4.4	Anticoagulant; inhibits tissue factor/factor VIIa complex

* Fold change values are shown for genes with a significant ($P < 0.05$) and >1.5-fold differential expression between samples of the medial tibial plateau (MTP) and lateral tibial plateau (LTP) from 10 patients with osteoarthritis (OA) and 10 cadaver donors (normal controls). Positive values represent greater expression in OA samples; negative values represent greater expression in normal samples. TGFβ = transforming growth factor β; EGF = epidermal growth factor; VEGF = vascular endothelial growth factor; IL-1β = interleukin-1β; TNFα = tumor necrosis factor α.

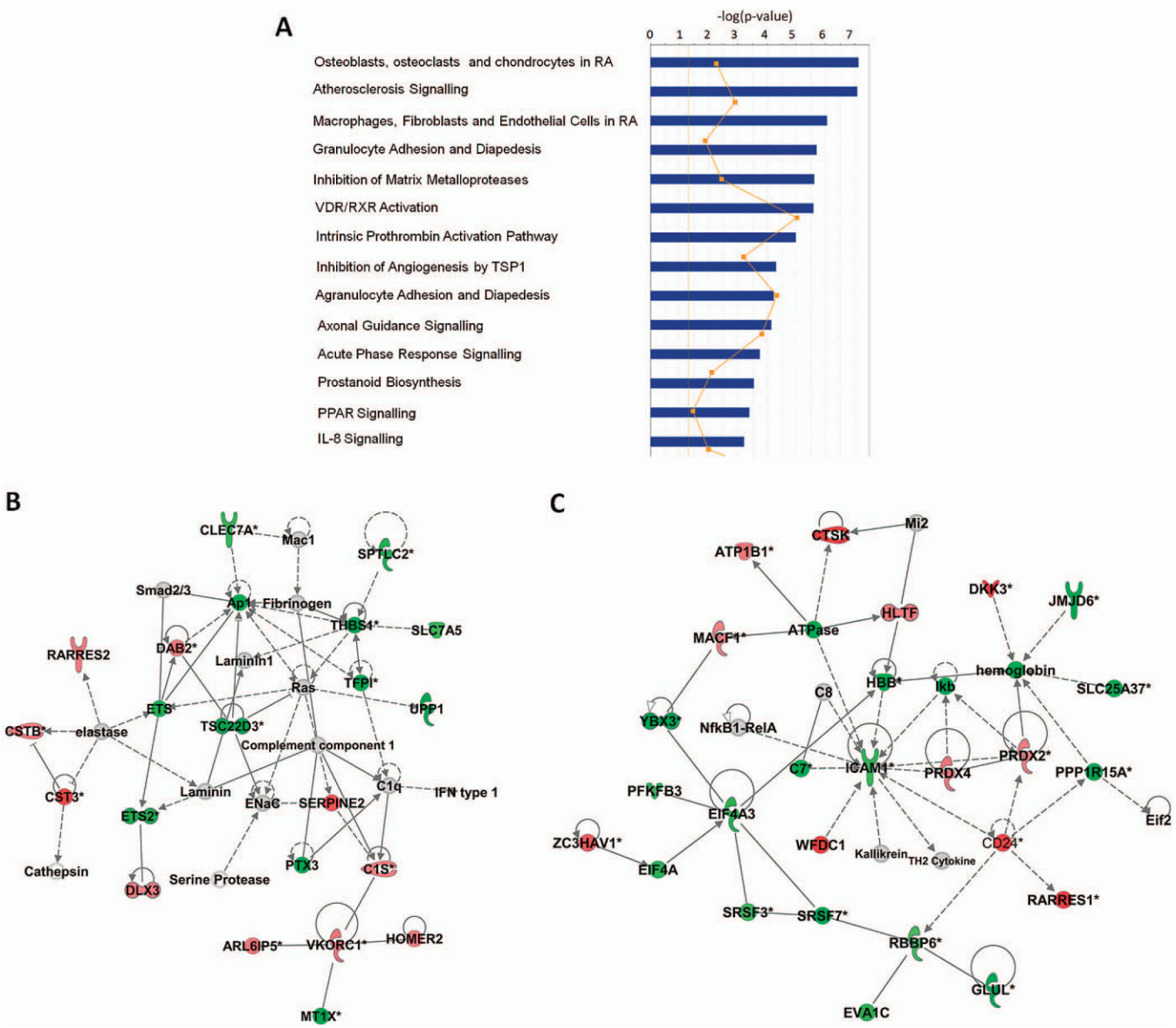

Figure 2. Pathway analysis of Affymetrix gene data. **A,** Core functional analysis using the Ingenuity Pathway Analysis (IPA) application. Shown are the most significantly differentiated pathways in osteoarthritic (OA) versus normal medial tibial plateau bone (using a cutoff of >1.5-fold differentiation and $P < 0.05$). The significance of the association of a given canonical pathway and the differentially expressed gene targets was measured in 2 ways: by the ratio of the number of genes in the network that mapped to the canonical pathway divided by the total number of genes that mapped to the canonical pathway (orange plot), and by using Fisher's exact test to calculate a *P* value for the association between the genes in the network and the canonical pathway (blue bars). Yellow vertical line indicates threshold of significance. RA = rheumatoid arthritis. **B** and **C,** Overlay analysis of the differentially expressed genes from the Affymetrix gene data with the coagulation and cardiovascular disease gene network (**B**) and the hematologic gene network (**C**). Red symbols represent genes that were up-regulated in OA versus normal bone; green symbols represent genes that were down-regulated in OA versus normal bone. Shaded symbols represent genes that were not differentially expressed between OA and normal bone. Open symbols represent nodes that are not shown in the microarray dataset but are included in the network based on previously known interactions. Different shapes represent different molecular classes. Solid lines represent direct interactions. Broken lines represent indirect interactions. **Arrows** that circle up and over the symbols represent self-interaction. **Open arrowheads** represent translocation. Asterisks indicate that the gene is represented in the microarray dataset by multiple identifers.

pronounced change was in the expression of adrenomedullin, a potent vasodilator, which was down-regulated 18-fold and 23-fold in the medial and lateral tibial plateaus of OA bone, respectively, as compared to normal tissue. In addition, both VEGFA and ANGPTL4, key genes that mediate angiogenesis, were

also down-regulated in OA bone. There was also increased expression of EDIL3 (2-fold), CTHRC1 (5-fold), and osteoglycin (3-fold), genes that have previously been associated with vascular injury and remodeling (24,25).

In addition, there was an ~4-fold down-regulation in the expression of the anticoagulant TFPI, a 2-fold down-regulation in the expression of SERPINE1, and increased expression of CD84, which mediates platelet aggregation. This suggests an altered balance between coagulation and fibrinolysis in OA bone tissue.

To further understand these findings, we uploaded our medial tibial plateau Affymetrix gene expression data to the IPA application (www.ingenuity.com) and performed a core functional analysis. The results of the canonical pathway analysis showed that from the library of canonical pathways, the most significant pathway was "role of osteoblasts, osteoclasts, and chondrocytes in RA." However, among the most significant pathways were "atherosclerosis signaling," "intrinsic prothrombin activation pathway," and "inhibition of angiogenesis by TSP1" canonical pathways (Figure 2A). In addition, the canonical pathway "role of macrophages, fibroblasts, and endothelial cells in arthritis" scored very highly where there was a high number of differentially expressed bone Affymetrix genes associated with endothelial cell signaling (data not shown).

We then performed an overlay analysis of our differentially expressed bone Affymetrix genes with specific gene networks and found a strong overlay of differentially expressed genes with both the "coagulation and cardiovascular disease" gene network (Figure 2B) and the "hematologic" gene network (Figure 2C).

Hypoxia-modulated expression of genes that mediate matrix integrity and bone remodeling and reduced activity of alkaline phosphatase. Culturing primary knee OA osteoblasts under conditions of hypoxia (2% O_2) stabilized the expression of HIF-1α, which became detectable within 4 hours (Figure 3A). After 24 hours, hypoxia had a profound effect on several genes purported to play a key role in bone remodeling, matrix integrity, and the bone vasculature (Table 2).

Hypoxia induced a significant 8.5-fold increase in the expression of MMP9 and a 16.2-fold increase in the expression of COL2A1, the gene that expresses the α1(II) chain of type II collagen. However, there was no significant effect on the expression of other bone matrix collagens (COL1A1, COL1A2, COL5A1, COL5A2, and COL13A). Hypoxia-treated osteoblasts also modulated the expression of key bone osteoblast transcription factors, which mediate osteoblast differentiation, with increased expression of GADD45B, but decreased expression of DLX5 and the osteoblast-specific transcription factor SP7 (osterix). In addition, hypoxia induced moderate increases in the expression of both RANKL and the decoy receptor OPG, which are

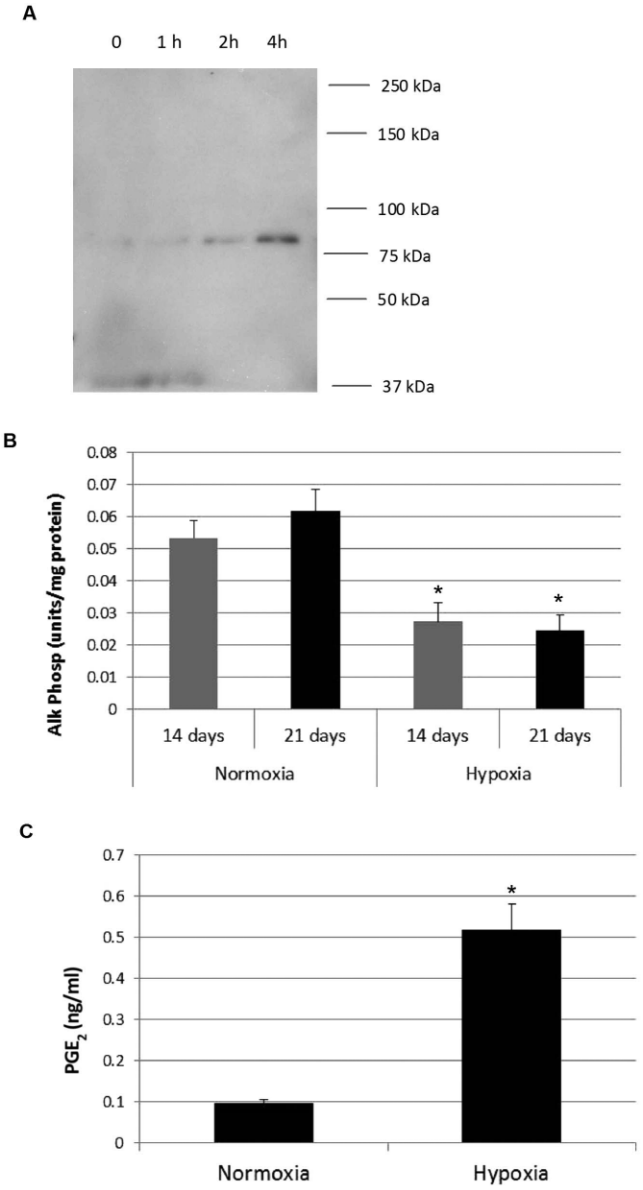

Figure 3. Hypoxia modulation of primary human osteoarthritic (OA) osteoblast alkaline phosphatase activity and prostaglandin E_2 (PGE_2) production. Primary human OA osteoblasts were cultured in a 24-well plate under hypoxic conditions (2% O_2). **A,** Expression of hypoxia-inducible factor 1α (HIF-1α) in OA osteoblasts. At 0, 1, 2, and 4 hours, osteoblasts were lysed using radioimmunoprecipitation assay buffer, and total cell lysates were immunoprobed with an anti–HIF-1α antibody. **B,** Alkaline phosphatase (alk phosp) activity in OA osteoblasts. At 14 and 21 days, cells were lysed, and the total protein and alkaline phosphatase activity were determined in the lysates. Values are the mean ± SEM (n = 6 samples per group). **C,** After 24 and 48 hours, supernatants were harvested and the levels of PGE_2 determined using a commercially available enzyme-linked immunosorbent assay. * = $P < 0.01$ versus the corresponding normoxic condition.

Table 2. Effect of hypoxia on the phenotype of primary human osteoblasts isolated from patients with knee OA*

Gene	Gene symbol	TaqMan assay ID	Fold change (hypoxia vs. normoxia)
Matrix remodeling			
Type II collagen α1	COL2A1	Hs00264051_m1	16.2
Matrix metalloprotease 9	MMP9	Hs00234579_m1	8.5
Osteoblast activity			
ADAM metallopeptidase domain 8	ADAM8	Hs00174246_m1	21.4
Growth arrest and DNA damage–inducible β	GADD45B	Hs00169587_m1	2.4
TNF receptor superfamily member 11b (OPG)	TNFRSF11B	Hs00171068_m1	2.0
TNF ligand superfamily member 11 (RANKL)	TNFSF11	Hs00243522_m1	2.1
Distal-less homeobox 5	DLX5	Hs00193291_m1	−2.0
Osterix	SP7	Hs00541729_m1	−10.5
Alkaline phosphatase liver/bone/kidney	ALPL	Hs00758162_m1	−1.6
Inflammation			
G protein–coupled receptor 68	GPR68	Hs00268858_s1	3.3
Interleukin-1β	IL1B	Hs00174097_m1	9.4
Prostaglandin endoperoxide synthase 2 (COX2)	PTGS2	Hs00153133_m1	2.0
Interleukin-6	IL6	Hs00174131_m1	−2.2
Chemokine (CXC motif) ligand 12	CXCL12	Hs00171022_m1	−1.8
Vasculature modulation			
Endothelin 1	EDN1	Hs00174961_m1	2.5
Vascular endothelial growth factor A	VEGFA	Hs00900054_m1	2.5
Angiopoietin-like 4	ANGPTL4	Hs00211522_m1	4.2
Angiopoietin 1	ANGPT1	Hs00266645_m1	−1.9
Antithrombin	SERPINC1	Hs00166654_m1	3.2
Plasminogen activator inhibitor type 1	SERPINE1	Hs00167155_m1	5.3
Tissue plasminogen activator	PLAT	Hs00263492_m1	5.6
Growth factor signaling			
Fibroblast growth factor 1	FGF1	Hs00265254_m1	2.7
Fibroblast growth factor 2	FGF2	Hs00266645_m1	2.2
Insulin-like growth factor binding protein 1	IGFBP1	Hs00236877_m1	4.2
Insulin-like growth factor binding protein 3	IGFBP3	Hs00181211_m1	2.8
Nerve growth factor β	NGFB	Hs00171458_m1	6.0

* Shown are genes with a significant ($P < 0.05$) and >1.5-fold differential expression in primary human osteoblasts derived from 4 osteoarthritis (OA) patients and cultured for 24 hours under conditions of hypoxia versus normoxia. TNF = tumor necrosis factor; OPG = osteoprotegerin; COX2 = cyclooxygenase 2.

known mediators of osteoblast/osteoclast coupling. There was also a reduction in the expression of ALPL in osteoblasts cultured under conditions of hypoxia, and this was reflected with reduced alkaline phosphatase activity, as determined by enzymatic assay, following culture of osteoblasts under conditions of chronic hypoxia (Figure 3B).

We also observed a hypoxia-induced elevation in the expression of several growth factor signaling components, with increased expression of FGF1, FGF2, and NGFB. Although hypoxia had no effect on the expression of IGF1 in osteoblasts, there was the suggestion that the IGF1 pathway was modulated, with the increased expression of the IGF1 binding proteins IGFBP1 and IGFBP3.

Hypoxia-modulated inflammatory cytokine and COX2 expression and induction of PGE_2 production. Hypoxia also modulated the expression of key inflammatory cytokines from osteoblasts, with a reduction in the expression of IL6 and CXCL12, but a 9-fold increase in IL1B expression. In addition, hypoxia led to a moderate yet significant increase (2-fold) in the expression of PTGS2 (COX2), suggesting activation of the arachidonic acid/COX2 pathway. Indeed, we observed elevated secretion of PGE_2 in the supernatants of hypoxia-treated osteoblasts as compared to normoxia-treated osteoblasts (Figure 3C).

Hypoxia-modulated expression of genes that mediate the vasculature. Given the evidence of elevated biomarkers of hypoxia in OA bone, we also examined whether hypoxia-stimulated primary OA osteoblasts showed altered expression of genes implicated in mediating the bone vasculature (Table 2). Importantly, we observed that hypoxia increased the expression of several genes known to be proangiogenic, namely, VEGFA and ANGPTL4, as well as elevating the expression of EDN1. In contrast, hypoxia significantly reduced the expression of ANGPTL1 as compared to normoxic conditions. Hypoxia also led to elevated expression of blood coagulation factors, with increased expression of the procoagulant SERPINE1 and increased expression of the anticoagulant PLAT.

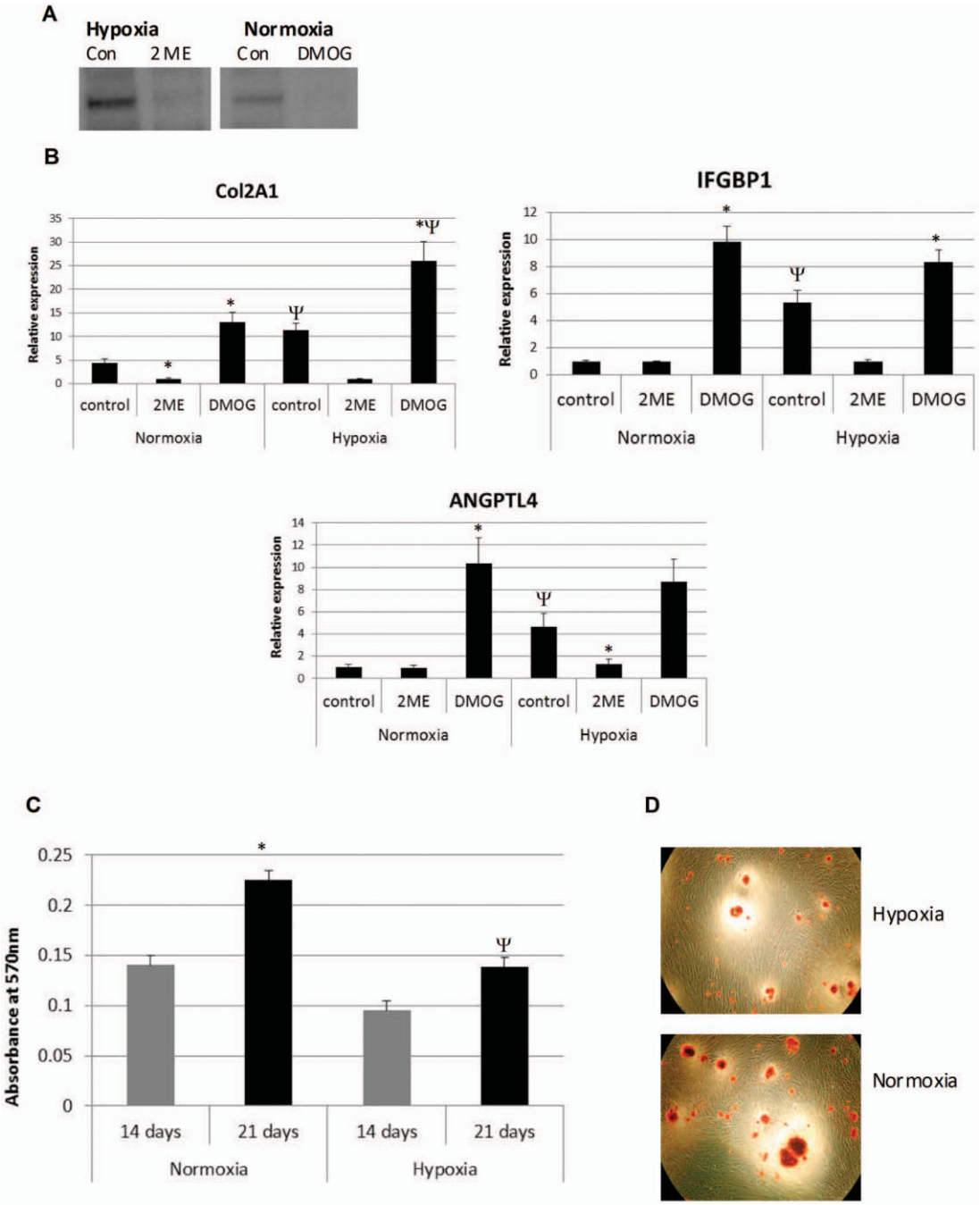

Figure 4. Hypoxia-inducible factor 1α (HIF-1α)–mediated expression of osteoblast genes and effect of chronic hypoxia on bone nodule formation. Primary osteoblasts were cultured in a 12-well plate (2×10^5 cells/well) under normoxic or hypoxic conditions and treated with either dimethyloxalylglycine (DMOG; 1 m*M*), 2-methoxyestradiol (2-ME; 200 μM), or DMSO (control) for 24 hours (n = 3 samples per condition). **A,** Protein lysates prepared with radioimmunoprecipitation assay buffer were immunoprobed for HIF-1α expression. **B,** Total RNA was extracted, and the expression of COL2A1, IGFBP1, and ANGPTL4 was quantified by quantitative reverse transcription–polymerase chain reaction analysis and normalized to 18S RNA. **C,** Confluent primary human OA osteoblasts were cultured for 14 or 21 days in 24-well plates under normoxic or hypoxic conditions. Bone nodules were then stained with alizarin red S, and the resulting stain was removed from the cells and released into the supernatant, and quantified by measuring its absorbance at 570 nm. Values in **B** and **C** are the mean ± SEM of 3 replicate experiments (**B**) or 3 separate donors (**C**). $* = P < 0.05$ versus the corresponding media alone control; $\Psi = P < 0.05$ versus the corresponding normoxic condition. **D,** Osteoblast bone nodules were cultured for 21 days under normoxic or hypoxic conditions and then stained with alizarin red S. Original magnification × 20. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.38403/abstract.

Mediation of osteoblast phenotype by modulation of HIF-1α levels. To examine whether pharmacologic modulation of HIF-1α levels in osteoblasts could mediate the expression of the hypoxia-regulated osteoblast genes we previously identified, osteoblasts were cultured for 24 hours under hypoxic or normoxic conditions, with or without dimethyloxalylglycine (DMOG; a cell-permeable prolyl-4-hydroxylase inhibitor which stabilizes HIF-1α) or 2-methoxyestradiol (2-ME; which down-regulates HIF-1α). First, we were able to demonstrate that, as expected, treatment of normoxic osteoblasts with DMOG (1 m*M*) stabilized HIF-1α expression, while conversely, treatment of hypoxic osteoblasts with 2-ME (200 μM) inhibited the induction of HIF-1α (Figure 4A). Then, we analyzed by qRT-PCR the effect of HIF-1α modulation on the expression of osteoblast hypoxia-regulated genes. The majority of the genes previously shown to be differentially expressed under hypoxic conditions were not significantly altered by >1.5-fold with either DMOG or 2-ME. However, the expression levels of ANGPTL4, COL2A1, and IGFBP1 were all partially regulated by pharmacologic modulation of HIF-1α using DMOG and 2-ME (Figure 4B).

Chronic hypoxia–inhibited osteoblast mineralization and bone nodule formation. To examine the effect of chronic hypoxia on bone nodule formation, we allowed primary OA osteoblasts to remain in culture in 24-well plates for 21 days, during which time they became highly confluent and formed bone nodules. Alizarin red S staining for mineralization (to enable visualization of bone nodules) showed that over this 21-day time course, hypoxia reduced the degree of mineralization as compared to that in osteoblasts cultured under normal oxygen conditions (Figures 4C and D).

DISCUSSION

In this study, we have provided important information on the role of hypoxia in OA bone pathology. This study is the first to show the effect of hypoxia on the phenotype of primary osteoblasts isolated from patients with knee OA. We also provide further evidence suggesting that subchondral bone tissue in OA patients is hypoxic by showing that subchondral bone tissue from patients with hip OA exhibits increased staining of a biomarker of hypoxia and that subchondral bone from patients with knee OA exhibits differential expression of genes associated with coagulation and vasculature pathways as compared to normal control tissue.

Hypoxia had a profound effect on both the activity and phenotype of primary OA osteoblasts isolated from patients with knee OA, which could play a key role in the development of sclerotic subchondral bone, a feature of OA. For example, hypoxia affected the expression of several osteoblast transcription factors that are known mediators of osteoblast differentiation, inducing an up-regulation in GADD45B expression and a down-regulation of both DLX5 and the osteoblast-specific transcription factor SP7 (osterix), which is considered to be a master regulator of osteoblast differentiation (26) and to be necessary for mineralization and bone formation (27–30). In addition, hypoxia reduced both the messenger RNA expression and protein activity of ALP, biomarkers of proliferative osteoblast activity.

Since it is known that a critical mass of differentiated osteoblasts is required for the formation of mature mineralized bone, we believe it is likely that both the hypoxia-induced down-regulation of the osteoblast-specific transcription factor SP7, together with a reduction in osteoblast proliferative activity played a role in the reduced mineralized bone nodule formation we observed in osteoblasts following treatment under conditions of chronic hypoxia, as compared to osteoblasts cultured under normoxic conditions.

These findings confirm in a human disease setting the previous observations of the effect of hypoxia on osteoblast proliferative activity and bone nodule formation using murine osteoblasts (22,23). Importantly, subchondral bone from OA patients is reported to be undermineralized (31) and may contribute to the subchondral bone sclerosis observed. As such, our finding that hypoxia reduces osteoblast mineralized bone nodule formation suggests that the hypomineralized OA bone could be driven by vascular remodeling and local hypoxia.

The formation of sclerotic subchondral bone in OA (1,2) has also been linked to alterations in growth factor signaling, particularly insulin-like growth factor 1 (32–35), as well as the urokinase plasminogen activator/plasminogen system (32,34), both of which may modulate the bone matrix. Of interest, we found that hypoxia-treated osteoblasts expressed higher levels of both PLAT and SERPINE1. Furthermore, although there was no effect of hypoxia on IGF1 expression, the expression of both IGFBP1 and IGFBP3 was elevated, suggesting that there was modulation of IGF1 signaling. Sclerotic OA bone has also been reported to contain altered expression of type I collagen genes (COL1A1 and COL1A2), leading to the formation of an abnormal type I collagen homotrimer (36). Furthermore, osteoblasts isolated from regions of sclerotic bone have been reported to express greater levels of both COL1A1 and COL1A2 compared to osteoblasts from nonsclerotic regions (37). In the present study, however, we found no effect of hypoxia on the osteoblast expression of type I collagen genes, but instead, we found a profound in-

crease in the expression of the type 2 collagen gene COL2A1, which is normally expressed predominantly in cartilage and at lower levels in bone tissue.

Our data also suggest that primary OA osteoblasts respond to hypoxia by elevating the expression of COX2 and producing greater amounts of PGE_2. Although we did not examine whether hypoxia affected EP receptor expression levels, it is known that osteoblasts express all 4 EP receptors. PGE_2 is considered a putative mediator of OA pain, and COX2 inhibitors have been shown to be analgesic in clinical studies. However, the role of PGE_2 in bone pathology is unclear. It was previously reported that PGE_2 levels increase during bone remodeling following fracture injury (38), and other investigators have shown that PGE_2 can stimulate osteoblast proliferation (39,40). Therefore, the hypoxia-induced elevation in PGE2 we report here could be an attempt by the osteoblasts to enhance proliferation rates.

Our data demonstrating that hypoxia modulates the osteoblast expression of several genes involved in the maintenance of the bone vasculature and endothelial cell integrity is intriguing. Previous studies (19,20) have demonstrated that OA bone exhibits signs of vascular obstruction, with reduced venous outflow. Importantly, evidence of neovascularization has been reported at the osteochondral junction in the OA joint (41). It is not established whether this neovascularization provides functional blood vessels, but it could be connected to our findings that OA subchondral bone shows signs of hypoxia, with neovascularization being a physiologic response to promote reoxygenation. In this context, the hypoxia-induced osteoblast expression of known mediators of angiogenesis (e.g., VEGF, ANGPTL4, ANGPT1, EDN1) may suggest that subchondral osteoblasts sense and respond to hypoxic microenvironments by mediating the local bone vasculature and may therefore play a central role in the neovascularization pathology previously reported within the OA osteochondral junction.

We also demonstrated that HIF-1α levels in primary OA osteoblasts can be modulated using the pharmacologic reagents DMOG and 2-ME, and that this translated into regulation of COL2A1, IGFBP1, and ANGPTL4 expression. However, pharmacologic modulation of HIF-1α levels did not lead to significant changes in all of the genes that we had previously shown were differentially expressed under hypoxic culture conditions. This could suggest the importance of other hypoxia-mediated factors, such as other HIF family members, or it could suggest that the effect of hypoxia on the expression of many of the genes was partly an indirect effect. For example, hypoxia induced a 9-fold increase in IL1B expression, and it has previously been found that IL1B can operate synergistically with hypoxia to elevate levels of VEGF in synovial fibroblasts (42) and articular chondrocytes (43). Another possibility is that multiple dosing of primary osteoblast cells with DMOG and 2-ME was required over the 24-hour period in order to achieve sufficient HIF-1α modulation to fully recapitulate the effect of hypoxia.

In summary, the findings of the present study show that the subchondral bone tissue of patients with OA exhibits signs of hypoxia and exhibits differential expression of genes that are known mediators of the vascular system as compared to normal bone obtained postmortem. Furthermore, we have shown that hypoxia can modulate the activity and phenotype of osteoblasts isolated from the tibia plateau bone of patients with knee OA, negatively affecting the degree of mineralized bone formation and modulating the expression of genes that mediate bone matrix integrity, bone remodeling, and bone vasculature. This suggests that identifying therapeutic approaches that can target local hypoxia within the subchondral bone may translate into effective disease modification by modulating osteoblast activity and preventing the formation of sclerotic undermineralized bone, which is a characteristic feature of OA.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Jones had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Wardale, Jones.

Acquisition of data. Chang, Jackson, Wardale, Jones.

Analysis and interpretation of data. Chang, Jackson, Wardale, Jones.

ROLE OF THE STUDY SPONSOR

AstraZeneca had no role in the study design or in the collection, analysis, or interpretation of the data, the writing of the manuscript, or the decision to submit the manuscript for publication. Publication of this article was not contingent upon approval by AstraZeneca.

REFERENCES

1. Buckland-Wright C. Subchondral bone changes in hand and knee osteoarthritis detected by radiography. Osteoarthritis Cartilage 2004;12 Suppl A:S10–9.
2. Burr DB, Gallant MA. Bone remodelling in osteoarthritis. Nat Rev Rheumatol 2012;8:665–73.
3. Radin EL. Subchondral bone changes and cartilage damage. Equine Vet J 1999;31:94–5.
4. Radin EL, Paul IL, Rose RM. Role of mechanical factors in pathogenesis of primary osteoarthritis. Lancet 1972;1:519–22.
5. Radin EL, Rose RM. Role of subchondral bone in the initiation and progression of cartilage damage. Clin Orthop Relat Res 1986;213:34–40.
6. Brandt KD, Radin EL, Dieppe PA, van de Putte L. Yet more evidence that osteoarthritis is not a cartilage disease. Ann Rheum Dis 2006;65:1261–4.

7. Anderson-MacKenzie JM, Quasnichka HL, Starr RL, Lewis EJ, Billingham ME, Bailey AJ. Fundamental subchondral bone changes in spontaneous knee osteoarthritis. Int J Biochem Cell Biol 2005;37:224–36.
8. Quasnichka HL, Anderson-MacKenzie JM, Bailey AJ. Subchondral bone and ligament changes precede cartilage degradation in guinea pig osteoarthritis. Biorheology 2006;43:389–97.
9. Hayami T, Pickarski M, Zhuo Y, Wesolowski GA, Rodan GA, Duong lT. Characterization of articular cartilage and subchondral bone changes in the rat anterior cruciate ligament transection and meniscectomized models of osteoarthritis. Bone 2006;38:234–43.
10. Neogi T. Clinical significance of bone changes in osteoarthritis. Ther Adv Musculoskelet Dis 2012;4:259–67.
11. Williams TG, Holmes AP, Waterton JC, Maciewicz RA, Hutchinson CE, Moots RJ, et al. Anatomically corresponded regional analysis of cartilage in asymptomatic and osteoarthritic knees by statistical shape modelling of the bone. IEEE Trans Med Imaging 2010;29:1541–59.
12. Marks R, Allegrante JP. Comorbid disease profiles of adults with end-stage hip osteoarthritis. Med Sci Monit 2002;8:CR305–9.
13. Conaghan PG, Vanharanta H, Dieppe PA. Is progressive osteoarthritis an atheromatous vascular disease? Ann Rheum Dis 2005;64:1539–41.
14. Weinberger M, Tierney WM, Booher P. Common problems experienced by adults with osteoarthritis. Arthritis Care Res 1989;2:94–100.
15. Rahman MM, Kopec JA, Anis AH, Cibere J, Goldsmith CH. Risk of cardiovascular disease in patients with osteoarthritis: a prospective longitudinal study. Arthritis Care Res (Hoboken) 2013;65:1951–8.
16. Rahman MM, Kopec JA, Cibere J, Goldsmith CH, Anis AH. The relationship between osteoarthritis and cardiovascular disease in a population health survey: a cross-sectional study. BMJ Open 2013;3:e002624.
17. Maes C, Carmeliet G, Schipani E. Hypoxia-driven pathways in bone development, regeneration and disease. Nat Rev Rheumatol 2012;8:358–66.
18. Hunter DJ, Gerstenfeld L, Bishop G, Davis AD, Mason ZD, Einhorn TA, et al. Bone marrow lesions from osteoarthritis knees are characterized by sclerotic bone that is less well mineralized. Arthritis Res Ther 2009;11:R11.
19. Arnoldi CC, Lemperg R, Linderholm H. Immediate effect of osteotomy on the intramedullary pressure in the femoral head and neck in patients with degenerative osteoarthritis. Acta Orthop Scand 1971;42:454–5.
20. Arnoldi CC, Linderholm H, Mussbichler H. Venous engorgement and intraosseous hypertension in osteoarthritis of the hip. J Bone Joint Surg Br 1972;54:409–21.
21. Pedersen NW, Kiaer T, Kristensen KD, Starklint H. Intraosseous pressure, oxygenation, and histology in arthrosis and osteonecrosis of the hip. Acta Orthop Scand 1989;60:415–7.
22. Warren SM, Steinbrech DS, Mehrara BJ, Saadeh PB, Greenwald JA, Spector JA, et al. Hypoxia regulates osteoblast gene expression. J Surg Res 2001;99:147–55.
23. Utting JC, Robins SP, Brandao-Burch A, Orriss IR, Behar J, Arnett TR. Hypoxia inhibits the growth, differentiation and bone-forming capacity of rat osteoblasts. Exp Cell Res 2006;312:1693–702.
24. LeClair R, Lindner V. The role of collagen triple helix repeat containing 1 in injured arteries, collagen expression, and transforming growth factor β signaling. Trends Cardiovasc Med 2007;17:202–5.
25. Kampmann A, Fernandez B, Deindl E, Kubin T, Pipp F, Eitenmuller I, et al. The proteoglycan osteoglycin/mimecan is correlated with arteriogenesis. Mol Cell Biochem 2009;322:15–23.
26. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, et al. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 2002;108:17–29.
27. Chen D, Li Y, Zhou Z, Xing Y, Zhong Y, Zou X, et al. Synergistic inhibition of Wnt pathway by HIF-1α and osteoblast-specific transcription factor osterix (Osx) in osteoblasts. PLoS One 2012;7:e52948.
28. Zhang C, Dai H, de Crombrugghe B. Characterization of Dkk1 gene regulation by the osteoblast-specific transcription factor Osx. Biochem Biophys Res Commun 2012;420:782–6.
29. Zhang C, Cho K, Huang Y, Lyons JP, Zhou X, Sinha K, et al. Inhibition of Wnt signaling by the osteoblast-specific transcription factor Osterix. Proc Natl Acad Sci U S A 2008;105:6936–41.
30. Zhang C. Transcriptional regulation of bone formation by the osteoblast-specific transcription factor Osx. J Orthop Surg Res 2010;5:37.
31. Cox LG, van Donkelaar CC, van Rietbergen B, Emans PJ, Ito K. Decreased bone tissue mineralization can partly explain subchondral sclerosis observed in osteoarthritis. Bone 2012;50:1152–61.
32. Hilal G, Martel-Pelletier J, Pelletier JP, Duval N, Lajeunesse D. Abnormal regulation of urokinase plasminogen activator by insulin-like growth factor 1 in human osteoarthritic subchondral osteoblasts. Arthritis Rheum 1999;42:2112–22.
33. Hilal G, Massicotte F, Martel-Pelletier J, Fernandes JC, Pelletier JP, Lajeunesse D. Endogenous prostaglandin E_2 and insulin-like growth factor 1 can modulate the levels of parathyroid hormone receptor in human osteoarthritic osteoblasts. J Bone Miner Res 2001;16:713–21.
34. Hilal G, Martel-Pelletier J, Pelletier JP, Ranger P, Lajeunesse D. Osteoblast-like cells from human subchondral osteoarthritic bone demonstrate an altered phenotype in vitro: possible role in subchondral bone sclerosis. Arthritis Rheum 1998;41:891–9.
35. Lajeunesse D, Hilal G, Pelletier JP, Martel-Pelletier J. Subchondral bone morphological and biochemical alterations in osteoarthritis. Osteoarthritis Cartilage 1999;7:321–2.
36. Truong LH, Kuliwaba JS, Tsangari H, Fazzalari NL. Differential gene expression of bone anabolic factors and trabecular bone architectural changes in the proximal femoral shaft of primary hip osteoarthritis patients. Arthritis Res Ther 2006;8:R188.
37. Sanchez C, Deberg MA, Bellahcene A, Castronovo V, Msika P, Delcour JP, et al. Phenotypic characterization of osteoblasts from the sclerotic zones of osteoarthritic subchondral bone [published erratum appears in Arthritis Rheum 2008;58:887]. Arthritis Rheum 2008;58:442–55.
38. Dekel S, Lenthall G, Francis MJ. Release of prostaglandins from bone and muscle after tibial fracture: an experimental study in rabbits. J Bone Joint Surg Br 1981;63-B:185–9.
39. Frost A, Jonsson KB, Nilsson O, Ljunggren O. Inflammatory cytokines regulate proliferation of cultured human osteoblasts. Acta Orthop Scand 1997;68:91–6.
40. Conconi MT, Tommasini M, Baiguera S, De Coppi P, Parnigotto PP, Nussdorfer GG. Effects of prostaglandins E1 and E2 on the growth and differentiation of osteoblast-like cells cultured in vitro. Int J Mol Med 2002;10:451–6.
41. Mapp PI, Walsh DA. Mechanisms and targets of angiogenesis and nerve growth in osteoarthritis. Nat Rev Rheumatol 2012;8:390–8.
42. Berse B, Hunt JA, Diegel RJ, Morganelli P, Yeo K, Brown F, et al. Hypoxia augments cytokine (transforming growth factor-β (TGF-β) and IL-1)-induced vascular endothelial growth factor secretion by human synovial fibroblasts. Clin Exp Immunol 1999;115:176–82.
43. Murata M, Yudoh K, Nakamura H, Kato T, Inoue K, Chiba J, et al. Distinct signaling pathways are involved in hypoxia- and IL-1-induced VEGF expression in human articular chondrocytes. J Orthop Res 2006;24:1544–54.