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Regulation of VEGF expression by HIF-1α in the femoral head cartilage following ischemia osteonecrosis

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Juvenile femoral head osteonecrosis is due to disruption of blood supply which results in ischemic injury. Angiogenesis is an essential component for the healing of damaged head. Hypoxia-inducible factor-1α (HIF-1α) is a master regulator of cellular response to hypoxia. Our histological studies showed increased vessel formation in cartilage in the ischemic group compared to the control group in a pig model of femoral head osteonecrosis. Microarray and RT-PCR indicated that VEGF expression was upregulated along with HIF-1α in the ischemic side. Immunohistochemistry assay demonstrated that HIF-1α and VEGF were upregulated in chondrocytes in ischemic femoral heads. Both HIF-1α and VEGF expression increased in primary chondrocytes under hypoxia station. Interestingly, an HIF-1α activator DFO further enhanced VEGF expression. Moreover, transfection of siRNA directed against HIF-1α led to inhibition of VEGF expression. Taken together, our data indicated that upregulation of VEGF during hypoxia in chondrocyte is mediated partially through HIF-1α.

Legg-Calve-Perthes disease (LCPD) is a common juvenile form of ischemic osteonecrosis of the femoral head that affects children between the ages of 2 to 14 years and has the attack rate of 1 in 740 boys and 1 in 3700 girls[1]. LCPD is due to blood supply disruption to the femoral head[2]. Ischemic osteonecrosis of the femoral head remains one of the most challenging conditions to treat due to the lack of understanding of the biology of the disease and our inability to modulate the repair process. Pig model of juvenile ischemic osteonecrosis of the femoral head has been shown to have radiographic and histopathologic changes resembling Legg-Calve-Perthes disease[3,4]. In the model, the induction of total femoral head ischemia produced extensive cell death in the hypertrophy zone of the epiphyseal cartilage and produced growth arrest of the secondary center of ossification, the bony epiphysis. In contrast, the more superficial region of the cartilage remained viable[5]. Angiogenesis is an essential component for the healing of damaged head. Our preliminary histological studies showed increased vessel formation in cartilage in the ischemic group compared to the control group in a pig model of femoral head osteonecrosis. The mechanism underlying this angiogenesis response to ischemic in the cartilage is not well known. Its significance to the repair process is an interesting area of research that may provide new insights into how we can stimulate revascularization and repair following femoral head ischemia.

Low oxygen tension or hypoxia is a pathophysiological component of many human diseases such as cancer, heart attack and stroke. Recent evidence suggests that it also plays a role in fetal skeletal development and cell differentiation[6,7]. The crucial mediator of the adaptive response of cells to hypoxia is the transcription factor, hypoxia-inducible factor-1 (HIF-1). HIF-1 is a heterodimer that consists of HIF-1α, the oxygen sensitive subunit, and the constitutively expressed HIF-1β. Under normoxic conditions, HIF-1α is hydroxylated by prolyl hydroxylases that act as oxygen sensors. Hydroxylation of specific proline residues on HIF-1α is followed by proteasomal degradation. Under hypoxic conditions, HIF-1α is stabilized, translocated to the nucleus, and forms a dimer with HIF-1β. HIF-1 activates target gene transcription by binding to the hypoxia-responsive elements in the proximal promoter region of the oxygen responsive genes. It has been reported that the HIF-1 complex is expressed in growth plate chondrocytes[8]. The growth plate is a constitutively avascular tissue in which the low oxygen partial pressure may impose energetic limitations on the cells as they progress from a proliferative to a terminally differentiated state. It has been shown that HIF-1α is essential for cell growth and survival of growth plate chondrocytes in vivo, as chondrocytes lacking functional HIF-1α undergo massive cell death in the center of the growth plate[9,10].

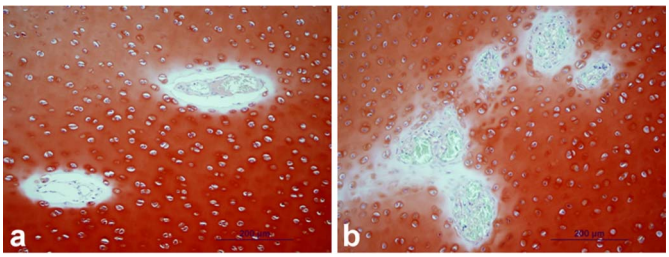

Figure 1 | The vessel formation increased after ischemia induction in pig femoral head. The femoral heads were obtained two weeks following the surgery to induce femoral head ischemia. Histological section of the epiphyseal cartilage from the control (a) and the ischemic sides (b) demonstrating increased vessel formation in cartilage in the ischemic side.

Vascular endothelial growth factor (VEGF) is an important mediator of angiogenesis. *VEGF* is expressed in the growth plate chondrocytes, and is thought to play a crucial role in regulating the number of blood vessels of the primary spongiosa[11]. When VEGF was inactivated in mice, it was found that the blood vessel invasion was nearly abolished, concomitant with the impaired trabecular bone formation and an expansion of the hypertrophic chondrocyte zone[11]. Studies conducted in different cell lines or in ES cell-derived tumors demonstrate that HIF-1α activity affects tumor cell growth by regulating both metabolic functions and VEGF expression[12–14]. However, the effect of HIF-1α on VEGF expression in chondrocytes following femoral head ischemia is not well understood.

In this study, we examined the coordinated expression of HIF-1α and VEGF in the cartilage in animal model of femoral head ischemia, and investigated the contribution of HIF-1α to VEGF upregulation in chondrocytes under hypoxic condition. We found that upregulation of VEGF during hypoxia in chondrocyte is mediated partially through HIF-1α.

Results

Vessel formation increased in femoral head after ischemia induction. In this study, ischemic osteonecrosis of the femoral head was surgically induced by applying a ligature tightly around the femoral neck and transecting the ligamentum teres. The femoral heads in the right side were operated as experiment groups and the unoperated left femoral heads were used as control groups. Animals were analyzed two weeks following the ischemia induction. Histologic assessments showed that vessel formation increased in cartilage in two weeks after ischemia induction as shown in Figure 1b compared with control group in Figure 1a in a pig model of femoral head osteonecrosis.

VEGF expression increased with HIF-1α in cartilage after ischemia induction. The mechanism underlying this vessel response in cartilage after ischemia induction is not well understood. To explore hypoxia-induced downstream gene activity following femoral head ischemia, porcine microarray was performed to compare gene expression profiles of chondrocytes from normal and ischemic femoral heads. We observed that expression of VEGF, a critical mediator of angiogenesis, was upregulated along with HIF-1α in the ischemic side in microarray (Data not shown). These microarray results were confirmed by quantitative real time RT-PCR. As shown in Figure 2, VEGF expression increased by 6.2 fold while HIF-1α expression increased by 2.9 fold in the ischemic side. These results indicated that VEGF activity increased after hypoxic injury to the femoral head.

To analyze HIF-1α and VEGF expression pattern in the cartilage, immunohistochemistry using antibodies against either HIF-1α or VEGF was performed in cartilage section isolated from ischemic and control groups two weeks after the surgery. As showed in Figure 3b, a significant increased expression of HIF-1α protein was

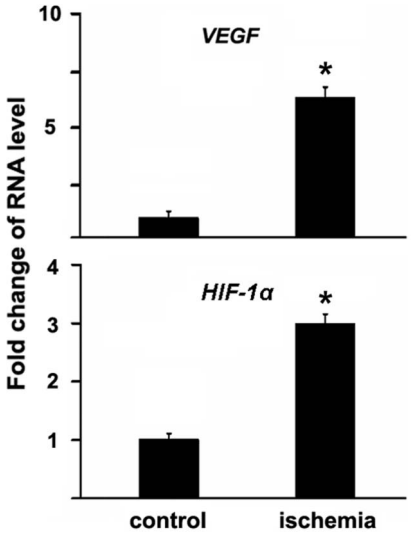

Figure 2 | Fold change in RNA levels of cartilage from ischemic and control group. RNA was isolated from cartilage 24 hr after ischemic induction in pig model. RNA levels were measured by real-time RT-PCR. Level of RNA from control group was normalized to a value of 1.

observed in the cartilage of ischemia group, compared with the cartilage of control group shown in Figure 3a. Percentage of HIF-1α positive cells in control and ischemic group was 6.0% and 47.5% respectively, indicating statistically significant increase in cartilage ($p<0.01$) as shown in Figure 3c. This result demonstrated that HIF-1α expression was upregulated after ischemia induction. At the same time, a significant higher expression of VEGF protein was also observed in the cartilage of the ischemic group shown in Figure 4b, compared with the cartilage of control group shown in Figure 4a. Percentage of VEGF positive cells in control and ischemic group was 6.3% and 38.2% respectively, indicating statistically significant increase in cartilage ($p<0.01$) as shown in Figure 4c. This observation demonstrated that VEGF expression was increased along with HIF-1α in the cartilage of ischemic femoral heads in this animal model after ischemia induction.

HIF-1α activated VEGF expression in chondrocytes after hypoxia. Although these findings show that both HIF-1α and VEGF expressions are increased after ischemia induction, the mechanism responsible for the upregulation of VEGF by hypoxia is not well understood in this animal model. HIF-1α is a crucial mediator of the adaptive response of cells to hypoxia, and essential for growth and survival of growth plate chondrocytes in vivo[9,10]. VEGF is an important mediator of angiogenesis. It has been shown that VEGF is required in both early and late stages of cartilage vascularization[15]. We hypothesize that hypoxia activates VEGF expression through HIF-1α after ischemia induction in this animal model. To test this hypothesis, we confirmed upregulation of both HIF-1α and VEGF under hypoxia in pig primary chondrocytes. Primary chondrocytes were isolated from cartilage of pig femoral heads. To examine the expression of VEGF and HIF-1α in vitro under hypoxia, pig primary chondrocytes were cultured for 24 hr in hypoxia condition with 1% or 20% of O_2. As shown in Figure 5a, expressions of HIF-1α and VEGF were upregulated by 2.5 fold and 5.4 fold respectively under hypoxia compared with control group.

To determine if hypoxia-induced VEGF expression is mediated by HIF-1α, we used gain-of-function approach to explore whether HIF-1α activators can enhance VEGF expression induced by hypoxia. Deferoxamine (DFO) is a known activator of HIF-1α. Primary chondrocytes were isolated from cartilage of pig femoral heads. As shown in Figure 5b, DFO treatment (100 uM) in vitro showed

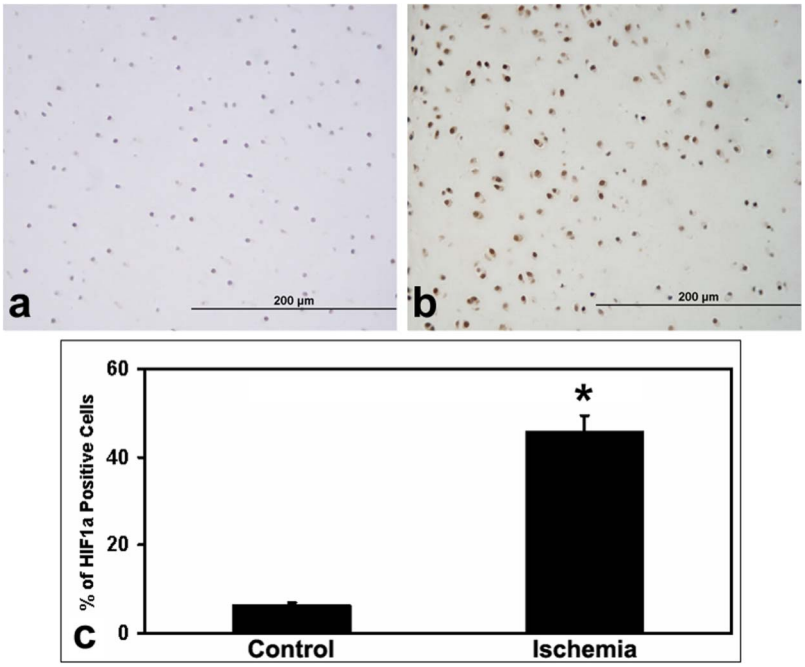

Figure 3 | HIF-1α expression increased in chondrocytes of ischemic group. HIF-1α immunostaining of epiphyseal cartilage two weeks after the surgery to induce ischemia. Increased immunostaining was observed in the ischemic side compared to the control side. Higher magnifications of control (a) and ischemic (b) are shown. Cells were counted at 40x magnification. At least 300 cells were counted and expressed as the percent of positive cells (c). A paired t-test was performed comparing the control and the contralateral ischemic sides.

further upregulation of VEGF expression by up to 9.2 fold under hypoxic condition (1% O_2), suggesting HIF-1α is involved in mediating VEGF expression in response to hypoxia.

Upregulation of VEGF during hypoxia is mediated partially through HIF-1α. After showing that HIF-1α activator can enhance VEGF expression induced by hypoxia, we next asked if HIF-1α is required for VEGF upregulation by hypoxia on the other hand. Loss-of-function approach was used to knockdown HIF-1α expression by siRNA directed against HIF-1α. HEK293 cell line was chosen for this approach because of its high transfection efficiency. Cells were transfected with siRNA directed against HIF-1α using Lipofectamine 2000 and incubated under hypoxia station. As shown in Figure 6, expression of VEGF was reduced by 44% after HIF-1α siRNA

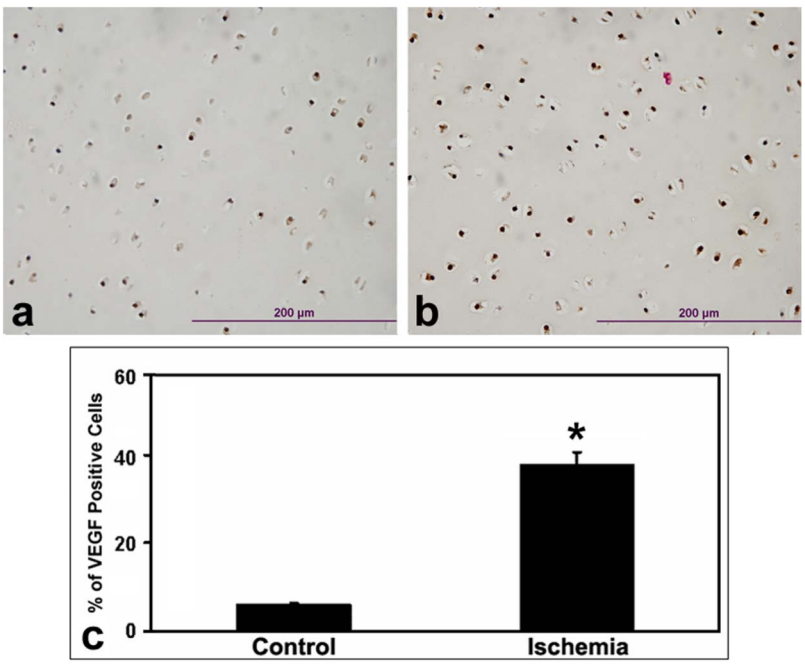

Figure 4 | VEGF expression increased in chondrocytes of ischemic group. VEGF immunostaining of epiphyseal cartilage two weeks after the surgery to induce ischemia. Increased immunostaining was observed in the ischemic side compared to the control side. Higher magnifications of control (a) and ischemic (b) are shown, respectively. Cells were counted at 40x magnification. At least 300 cells were counted and expressed as the percent of positive cells (c). A paired t-test was performed comparing the control and the contralateral ischemic sides.

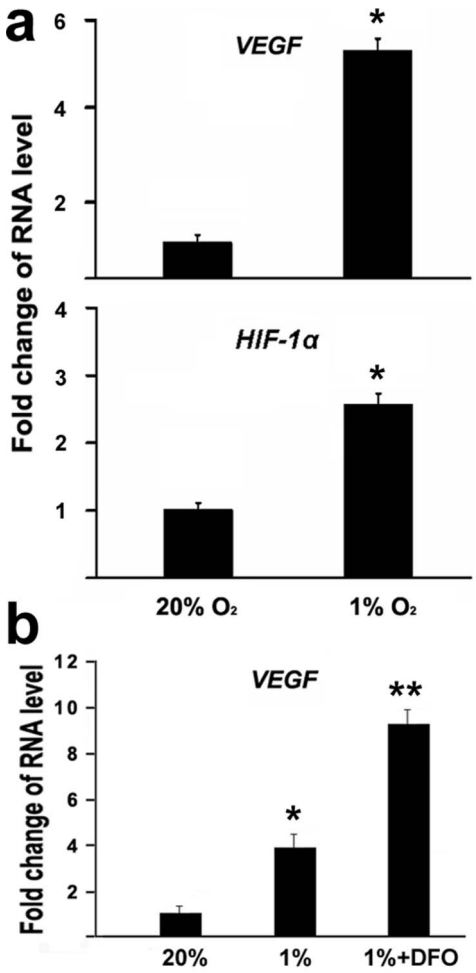

Figure 5 | Fold change in RNA levels of chondrocytes under hypoxia. Primary chondrocytes were isolated from cartilage of pig femoral heads. Pig primary chondrocytes were cultured for 24 hr in hypoxia station with 1% or 20% of O^2. RNA levels were isolated and measured by real-time RT-PCR for expression of VEGF and HIF-1α (a). Level of each type of RNA from control group was normalized to a value of 1. (b) DFO enhanced VEGF expression under hypoxia. Pig primary chondrocytes were cultured in normal ($21\%O_2$) and hypoxia ($1\%O_2$) condition. In one group of hypoxia condition, HIF-1α activator DFO was added at 100 μM. RNA was isolated 24 hr after hypoxia. RNA levels were measured by real-time RT-PCR. Level of each type of RNA from control group was normalized to a value of 1. *: A star indicates statistical significance compared to expression level in normal condition ($p<0.05$, N=3). **: Two stars indicate statistical significance compared to expression level in hypoxia condition in the absence of DFO ($p<0.05$, N=3).

transfection (Figure 6b) while expression of HIF-1α was inhibited by 81% (Figure 6a). On one hand, expression of VEGF was reduced after siRNA transfection against HIF-1α, suggesting that upregulation of VEGF during hypoxia is mediated through HIF-1α. On other hand, we found that expression of VEGF was reduced significantly but not abolished after HIF-1α siRNA transfection, suggesting that upregulation of VEGF during hypoxia is mediated partially through HIF-1α.

Discussion

Legg-Calve-Perthes disease is a juvenile form of ischemic osteonecrosis of the femoral head that can produce femoral head deformity and premature osteoarthritis. It remains one of the most challenging conditions to treat due to our lack of understanding of the biology of the disease. Femoral head osteonecrosis occurs due to blood supply disruption which produces hypoxic injury to the femoral head. Our histological studies showed increased vessel formation in cartilage in the ischemic group compared to the control group in a pig model of femoral head osteonecrosis. Here we have presented evidence to indicate that there are coordinated expressions of HIF-1α and VEGF in the cartilage in animal model of femoral head ischemia and that upregulation of VEGF during hypoxia in chondrocyte is mediated partially through HIF-1α.

First, we identified coordinated upregulation of HIF-1α and VEGF under hypoxia in RNA expression level in the ischemic femoral heads. This is supported by the cartilage and primary chondrocyte analysis. Quantitative RT-PCR confirmed Microarray results to show that the RNA expression of VEGF was upregulated along with HIF-1α in pig ischemic cartilage (Figure 2). Coordinated upregulation of HIF-1α and VEGF under hypoxia was also observed in primary chondrocytes isolated from femoral heads (Figure 5a). Our immunohistochemistry assay indicated that both HIF-1α and VEGF were upregulated in chondrocytes in the ischemic femoral heads also at the protein level in Figure 3 and Figure 4. To investigate the mechanisms responsible for upregulation of VEGF by hypoxia, we tested the effects of HIF-1α activator, DFO, on VEGF expression in chondrocytes under hypoxia. Results from this study indicate that DFO enhanced VEGF expression under hypoxia (Figure 5b), suggesting HIF-1α is involved in mediating VEGF expression in chondrocytes under hypoxia.

Conditional HIF-1α knockout mice provide strong evidence for the involvement of HIF-1α in cartilage development. In these animals, HIF-1α deletion in chondrocytes in the interior of the developing growth plate induced premature programmed cell death. In addition, chondrocytes lacking HIF-1α exposed to hypoxia showed decreased expression of chondrocyte marker gene Col2a[10]. This in vitro study provides partial molecular mechanisms for transcriptional activity of HIF-1α during hypoxia. VEGF is a well-characterized angiogenic factor that is activated by hypoxia. HIF-1α is a master regulator of cellular response to hypoxia. The loss of HIF-1α makes bone narrow and less vascularized. Nevertheless, VEGF was still expressed in HIF-1α null mice indicating that besides HIF-1α, other factors are also involved in VEGF regulation during embryonic development[16]. Our observation in current study is consistent with this notion from the HIF-1α null mice data in that expression of VEGF was reduced significantly but not abolished after HIF-1α siRNA transfection as shown in Figure 6. VEGF expression was reduced by 44% after HIF-1α knockdown by siRNA transfection, suggesting that 1) HIF-1α is involved in VEGF upregulation during hypoxia and that 2) other factors besides HIF-1α may also be responsible for VEGF regulation during hypoxia. HIF-1α upregulation of VEGF activity may be one of the mechanisms for angiogenesis response following femoral head ischemia.

It is still an open question what other factors control VEGF expression besides HIF-1α. Runx2 is an essential regulator of both endochondral and intramembranous ossification[17]. Runx2 has been shown to control VEGF expression in chondrocytes during endochondral bone formation[18]. There is no VEGF expression in the hypertrophic chondrocytes of Runx2 knockout mice. Overexpression of Runx2 in fibroblasts induces an increase in VEGF mRNA and protein levels by upregulating VEGF transcription[18]. It is possible that Runx2 is also involved in VEGF regulation in the absence of HIF-1α. Osterix (Osx) is an osteoblast-specific transcription factor required for bone formation, and is considered a master regulator essential for the commitment of preosteoblast differentiation into mature osteoblasts[19,20]. Osx directly targets VEGF expression, involving direct binding of Osx to sequence specific promoter elements to activate the VEGF expression in osteoblasts[21]. It has been demonstrated that Osx regulation of VEGF is independent of HIF-1α expression level[22]. Interestingly, Osx cooperates with HIF-1α to positively regulate VEGF expression[22]. VEGF regulations by HIF-1α–independent mechanisms deserve further investigation.

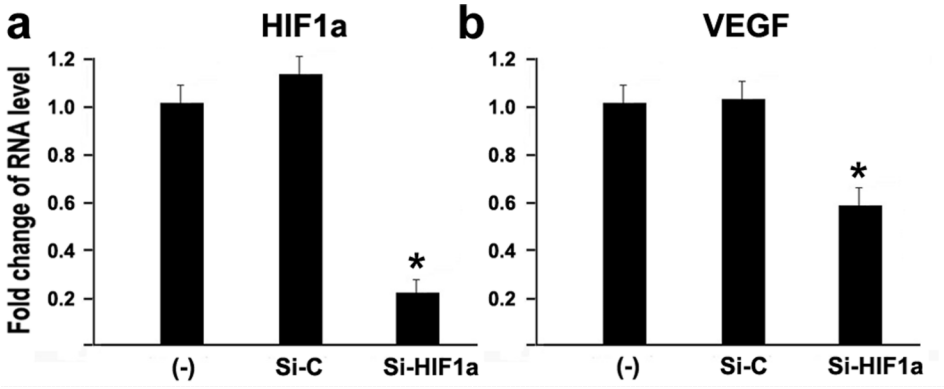

Figure 6 | Upregulation of VEGF during hypoxia is mediated partially through HIF-1α. RNA expression levels of HIF-1α (a) and VEGF (b) as determined by quantitative real-time RT-PCR. HEK293 cells were transfected with siRNA targeting HIF-1α, and then cultured in hypoxia station. RNA was isolated in 24 hr and quantitated by real-time RT-PCR. The RNA level from the control group was normalized to a value of 1. Values were presented as the mean ±S.D.

In summary, we have demonstrated that upregulation of VEGF in chondrocytes following femoral head ischemia is mediated partially through HIF-1α. Ischemic osteonecrosis of the femoral head is a significant clinical problem that can lead to femoral head collapse and early degenerative arthritis in young patients[23]. Our understanding of the pathogenesis and repair process is still much limited. Results in this study provide the possible mechanisms to explain the more vessel formation after ischemia induction in pig femoral head via VEGF regulation partially through HIF-1α. Further studies are needed to identify additional factors which may be responsible for more vessel formation after ischemia induction in this model.

Methods

Animal and cartilage isolation. The animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center. Yorkshire immature pigs aged 5 to 6 weeks old (6 to 8 kg) were used. Ischemic osteonecrosis of the femoral head was surgically induced by applying a ligature tightly around the femoral neck and transecting the ligamentum teres, as previously described[3]. Only the right side was operated and the unoperated left femoral heads were used as controls. The animals were euthanized 24 hr or two weeks as indicated following the ischemia induction.

Histology and immunohistochemistry. As previously described[24], Femoral heads were cut into four-millimeter thick sections in a coronal plane using Isomet 1000 precision saw (Buehler Ltd., Lake Bluff, IL), fixed in 10% neutral buffered formalin, decalcified using ethylenediamine tetra acetic acid (EDTA), and embedded in paraffin. Five-micron sections were cut and mounted on aminosaline adhesive coated slides. Sections were stained with hematoxylin-eosin or safranin O-fast green stains. For immunohistochemistry, deparaffinized sections were digested with 0.05% trypsin in 50 Mm Tris/HCL buffer at room temperature for 15 minutes and washed with distilled water. Endogenous peroxidase activity was deactivated using 3% H_20_2 in distilled water for 10 minutes at room temperature and rinsed several times in 1xTBS. Sections were blocked in (ImmunoVision Technologies,Co) pre-antibody blocking/diluent solution for 1 hr at room temperature to reduce nonspecific binding and incubated overnight at 4°C with a VEGF primary antibody in a (1 : 1000) pre-blocking diluent. A rabbit polyclonal antibody (Abcam) against HIF-1α was used as a primary antibody. A Post blocking polymer penetration enhancer (ImmunoVision Technologies,Co) and Poly-HRP anti Mouse/Rabbit IgG (ImmunoVision Technologies,Co) was used. The substrate, DAB (ImmunoVision Technologies,Co), was placed on the sections for 6 minutes. The sections were dehydrated in alcohol and mounted using permount. Controls were run in parallel by replacing the primary antibody with only incubating buffer or by adding the same concentration of IgG. All sections were examined under a Nikon Eclipse E800M Microscope at various magnifications. Digital images were obtained using a SPOT Digital Camera (Diagnostic Instruments) and the accompanying SPOT software. Cells were counted at 40x magnification in the mid-thickness of the cartilage. In each image, the number of cells with positive immunostaining was obtained, as well as the total number of cells present. At least 300 cells were counted and expressed as the percent of positive cells. A paired t-test was performed comparing the normal and the contralateral ischemic sides.

RNA isolation and microarray. Femoral heads were retrieved and cartilage samples were removed from articular cartilage of the femoral head using a sharp knife at the level of the cartilage-bone junction and snap frozen using liquid nitrogen. Total RNA was extracted using Trizol reagent (Invitrogen) following the manufacturer's protocol. Isolated RNA was treated with DNase I (Ambion) and purified by Qiagen RNeasy Mini Column (Qiagen). RNA concentration was determined using the Nanodrop ND 1000 Spectrophotometer. The integrity of the RNA was checked with the Agilent Bioanalyzer 2100 (Agilent). All RNA samples had an RNA Integrity Number above 7. Affymetrix GeneChip Porcine Genome Array containing 23,937 probe sets to interrogate 23,256 transcripts in pigs (20,201 genes) was used to compare gene expression profiles between Ischemic group and control group.

Quantitative real-time reverse transcription-PCR. RNA was subjected to quantitative RT-PCR using the TaqMan One-Step RT-PCR Master Mix reagent (Applied Biosystems) as previously described[25]. RT-qPCR was performed on a Thermal Cycler (iCycler, Bio-Rad) for 40 cycles at 95°C for 15 s and at 48 to 58°C, depending on the melting temperatures of the primers, for 30 seconds. Relative transcript levels were measured by real-time PCR in a 50 μl reaction volume on 96-well plates using an ABI PRISM 7000 sequence detection system (Applied Biosystems). Heat-shock protein 90 (Hsp-90) was tested for the stability of the expression on the ischemic side compared to the normal side and was used to normalize the expression levels of various genes studied using primers from Applied Biosystems.

Primary chondrocyte isolation and hypoxia experiment. Primary chondrocytes were isolated from cartilage of pig femoral heads. Briefly, cartilage from the femoral heads was cut into small pieces (2–3 mm^3), washed with Dulbecco's modified Eagle's medium (DMEM), and treated for 15 min with trypsin (10% v/v) in a 37°C water bath. Cartilage pieces were then transferred to DMEM containing 5% fetal calf serum, penicillin-streptomycin-Fungizone, and 2 mg/ml clostridial collagenase type IV and digested overnight on a shaker until the tissue fragments were dissolved. The cells were washed three times with DMEM and cultured in DMEM plus 10% fetal calf serum until confluent. All experiments used chondrocytes in the primary culture or at passage 1 following a 1 : 3 subculture. In hypoxia experiments, chondrocytes were cultured in DMEM and maintained in normoxic ($20\%O_2$) or hypoxia ($1\%O_2$) condition with $5\%CO_2$ and the balanced N_2 using a humidified hypoxia workstation (Coy Laboratories). All endpoints measured in hypoxia cells were compared with those in cells kept under normoxic condition.

siRNA interference. HEK293 cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum and 100 units/ml penicillin plus 100 μg/ml streptomycin at 95% air/5% CO_2 humidified incubator. HEK293 cells were transfected with siRNA directed against HIF-1α using Lipofectamine 2000. siRNA oligos were purchased from Thermo Scientific Dharmacon, and siGENOME Lamin A/C Control siRNA was used as a non-specific control. Cells were cultured in 6-well plates. Cells were plated in 1 ml of growth medium without antibiotics 1 day prior to transfection and the cells were 30–50% confluent at the time of transfection. The final siRNA concentration was 100 nM and each well received 100 μl of the siRNA:Lipofectamine. 2000 complex in Opti-MEM I medium.

Statistical analysis. All experiments were carried out with a minimum of n=3. We reported data as the mean with standard error. For histology studies, two histology sections from the central region of femoral head were used, and 3 pigs each group were analyzed. Comparisons were made between groups of equal size by Student's t test with $p<0.05$ considered statistically significant.

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Author contributions

Conceived and designed the experiments: CZ and HK. Performed the experiments: YL, RC, SS and HK. Analyzed the data: CZ and HK. Wrote the paper: CZ. CZ takes responsibility for the integrity of the data analysis.

Additional information

Competing financial interests: The authors declare no competing financial interests.

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