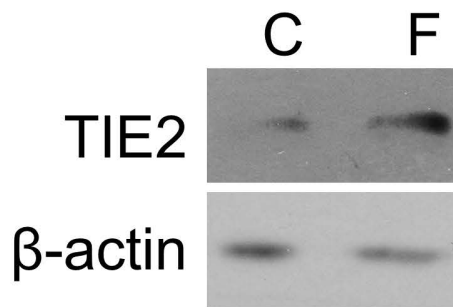
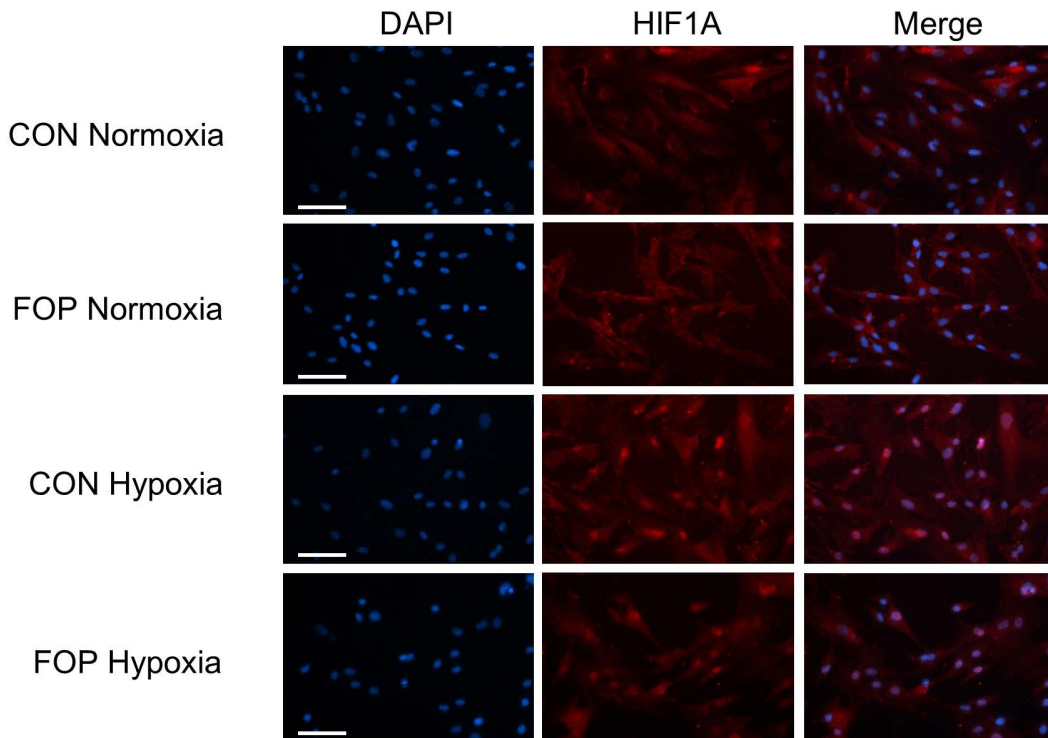


Injury triggers hypoxia in lesions from non-hereditary traumatic HEO. Representative images of nuclear localization of HIF-1 α by immunohistochemistry in a tissue section from an individual with traumatic HEO is shown. The lesion depicts the fibro-proliferative stage that typically precedes neovascularization and endochondral ossification. Scale bar, 50 μ m.

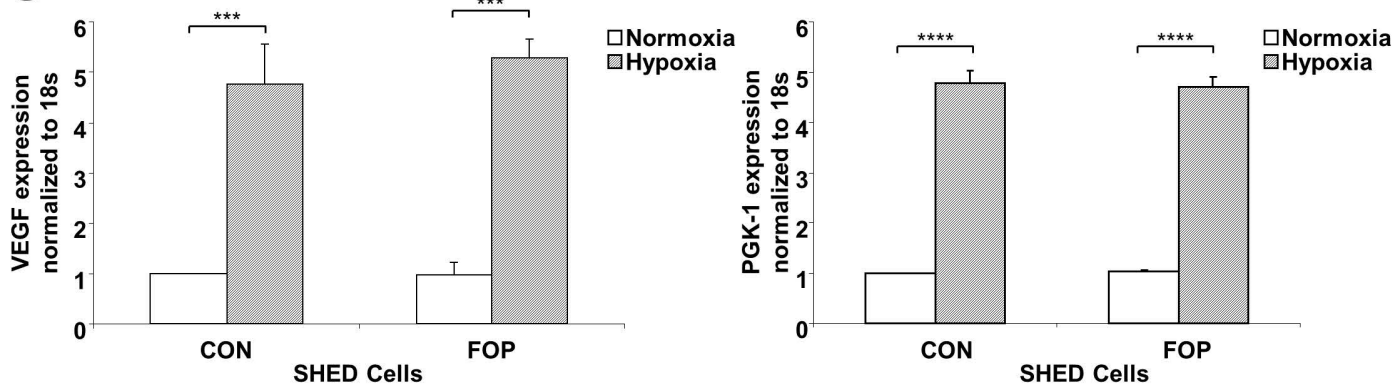
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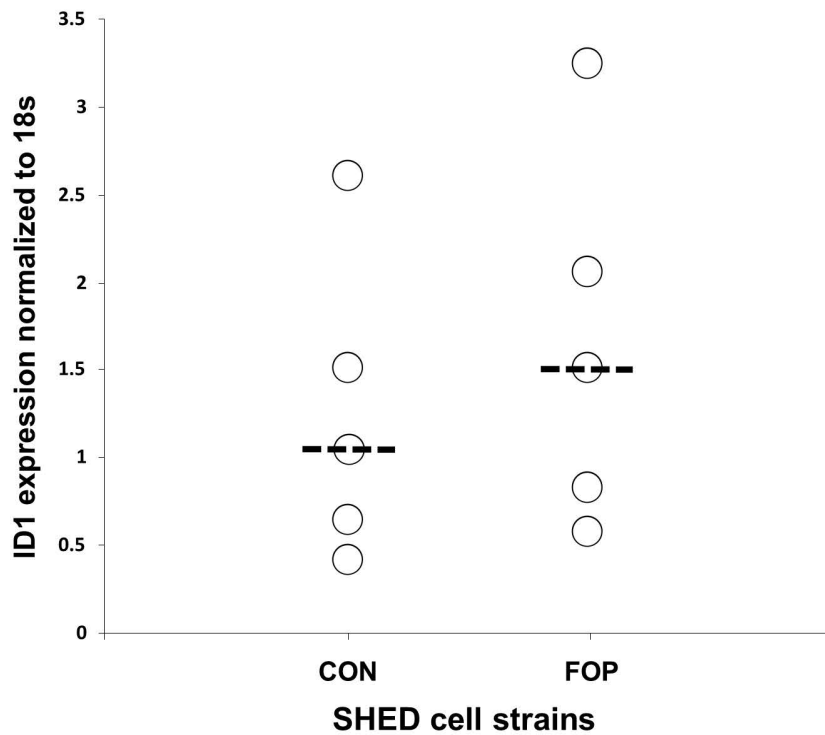
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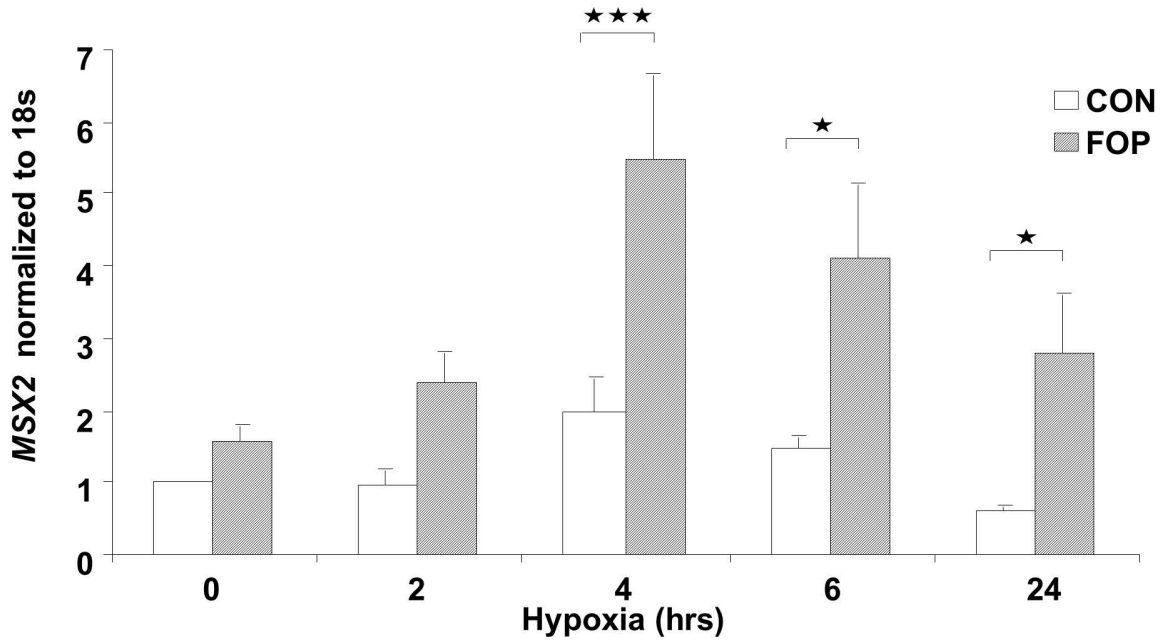
C



Characterization of SHED cells. (a) SHED cells are Tie2-positive by Western blot analysis. (b) Hypoxia promotes nuclear translocation of HIF-1 α in SHED cells. Immunofluorescence of HIF-1 α , DAPI, and merged images demonstrates localization of HIF-1 α to the nucleus at low oxygen tension in SHED cells. Scale bar = 50 μ m. There were no differences in HIF-1 α translocation into the nucleus between 5% and 1% O₂ (data not shown). (c) Enhanced expression of HIF-1 α target genes, VEGF and PGK-1 by real-time PCR. Hypoxia, but not genotype is significantly related to of HIF-1 α target gene expression ($p < 0.0001$) Bonferroni post-hoc analysis: ****, $p < 0.0001$; ***, $p < 0.001$; $n=3$.

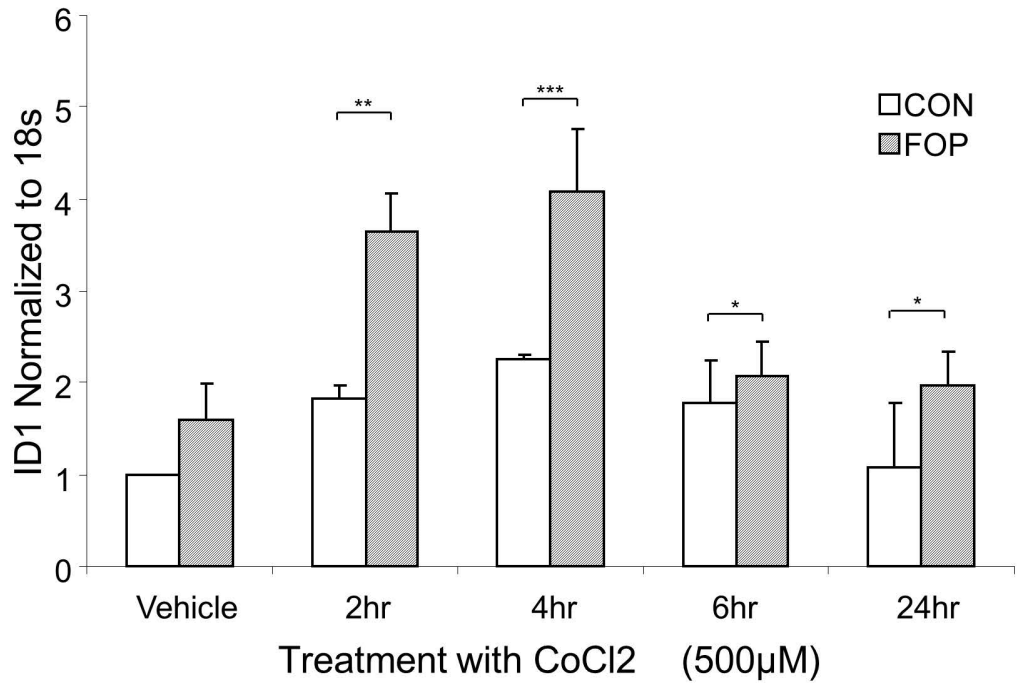


ID1 expression in 10 SHED cell strains under basal conditions (normoxia). Near-confluent cultures were washed of serum-containing medium and then serum-starved for 2 hours before harvesting mRNA for real-time PCR analysis. Control SHED cell strains were derived unaffected individuals aged 6.68 to 11.54 years. FOP SHED cell strains were derived from individuals aged 5.87 to 11.33 years. ----, median ID1 expression.

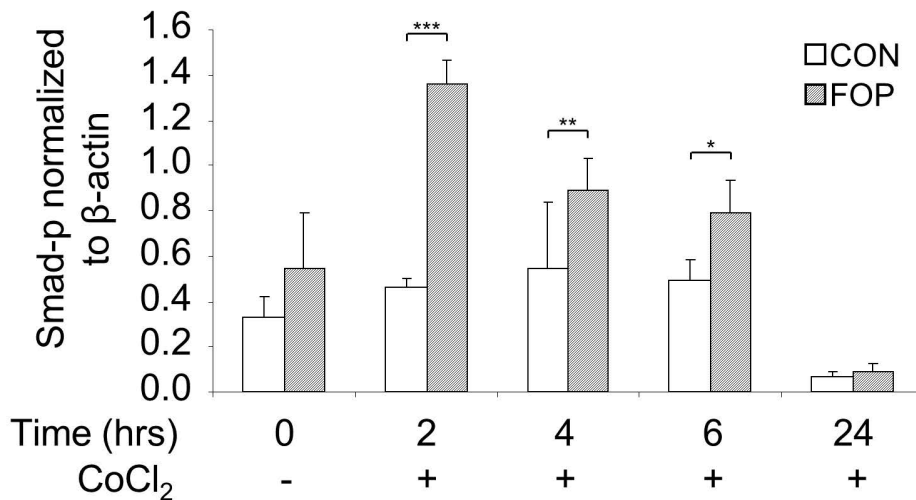
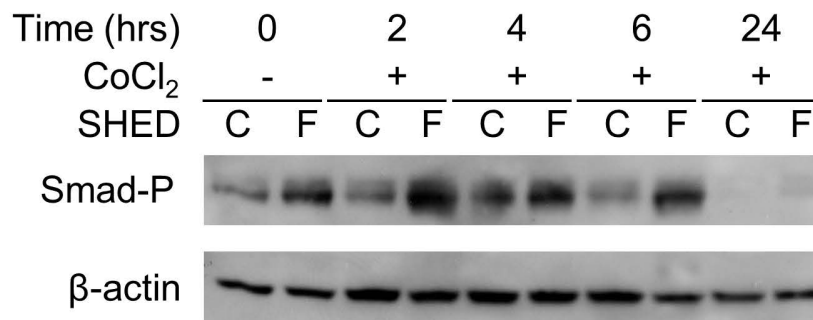


Relative *MSX2* expression in SHED cells under hypoxia. *MSX2* levels were quantified by real-time PCR and normalized to 18S RNA. Genotype and hypoxia are significantly related to *MSX2* expression by 2-way ANOVA ($p < 0.0001$ and $p < 0.001$, respectively). Bonferroni post-hoc analysis: ***, $p < 0.001$; *, $p < 0.05$; $n = 6$.

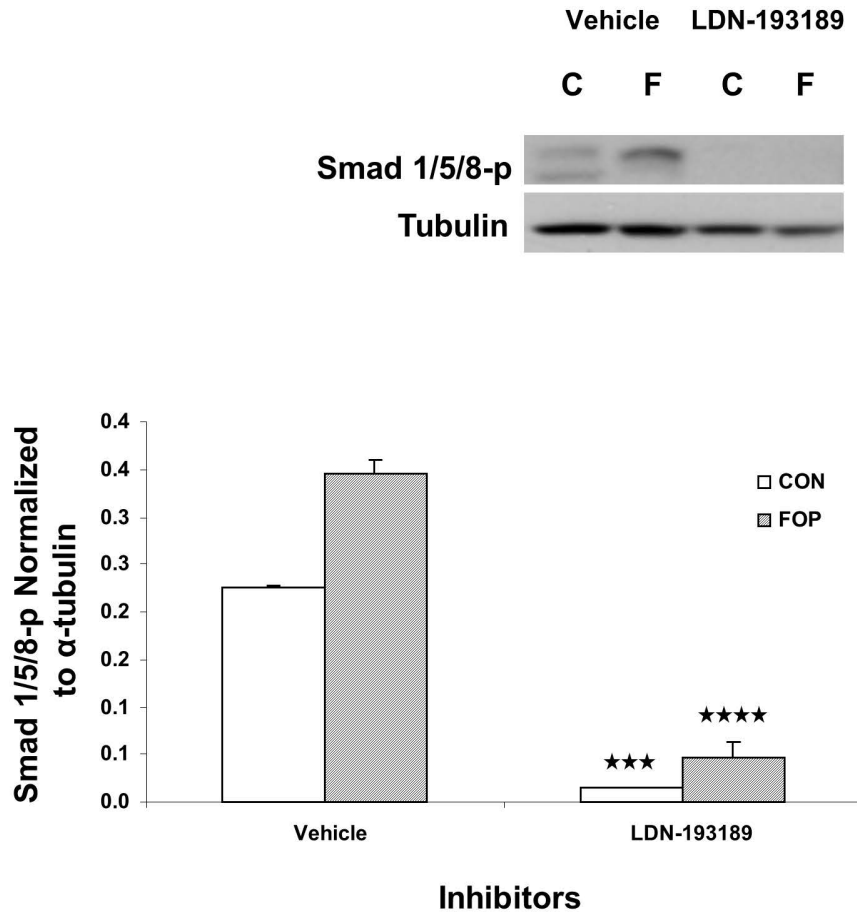
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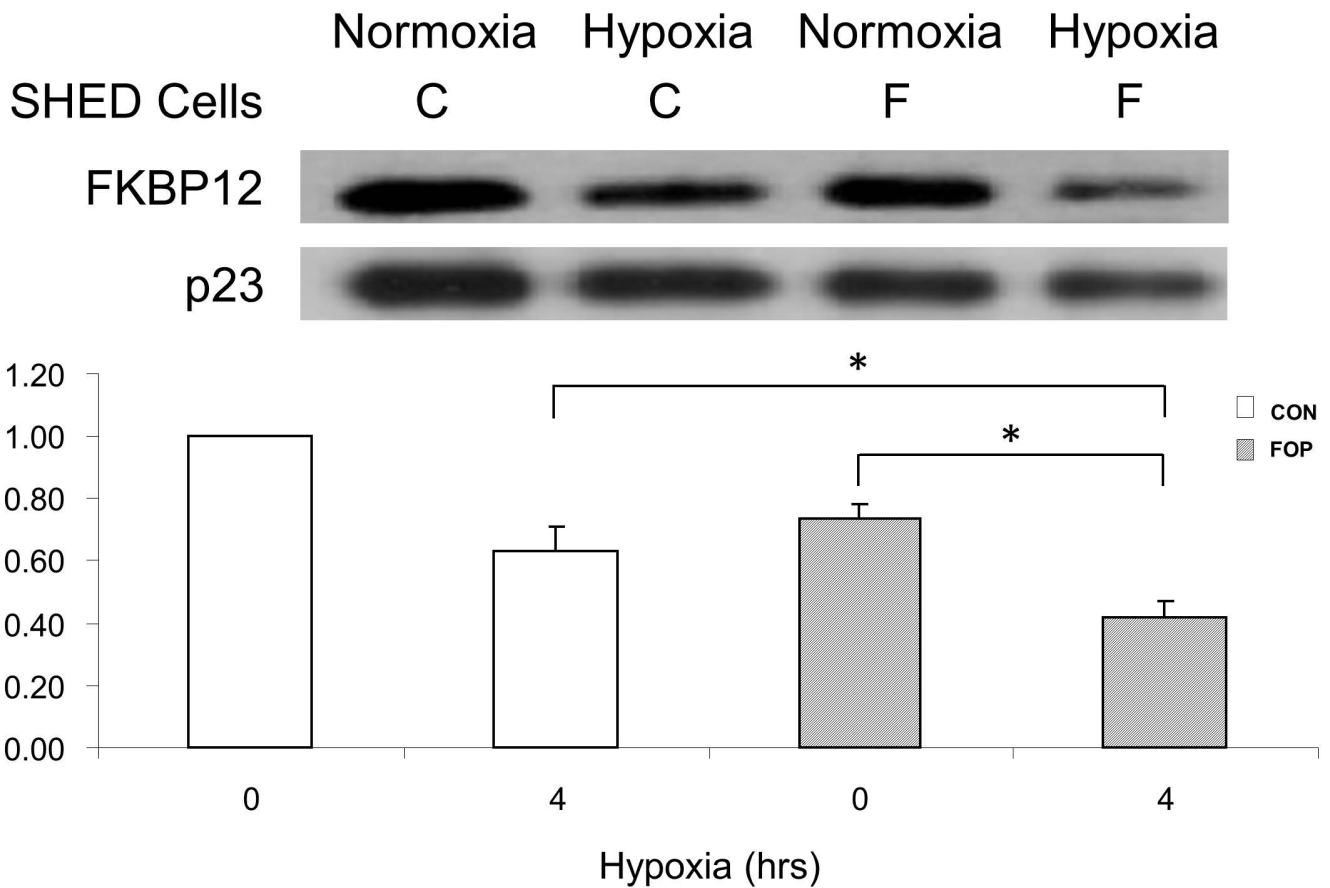
B



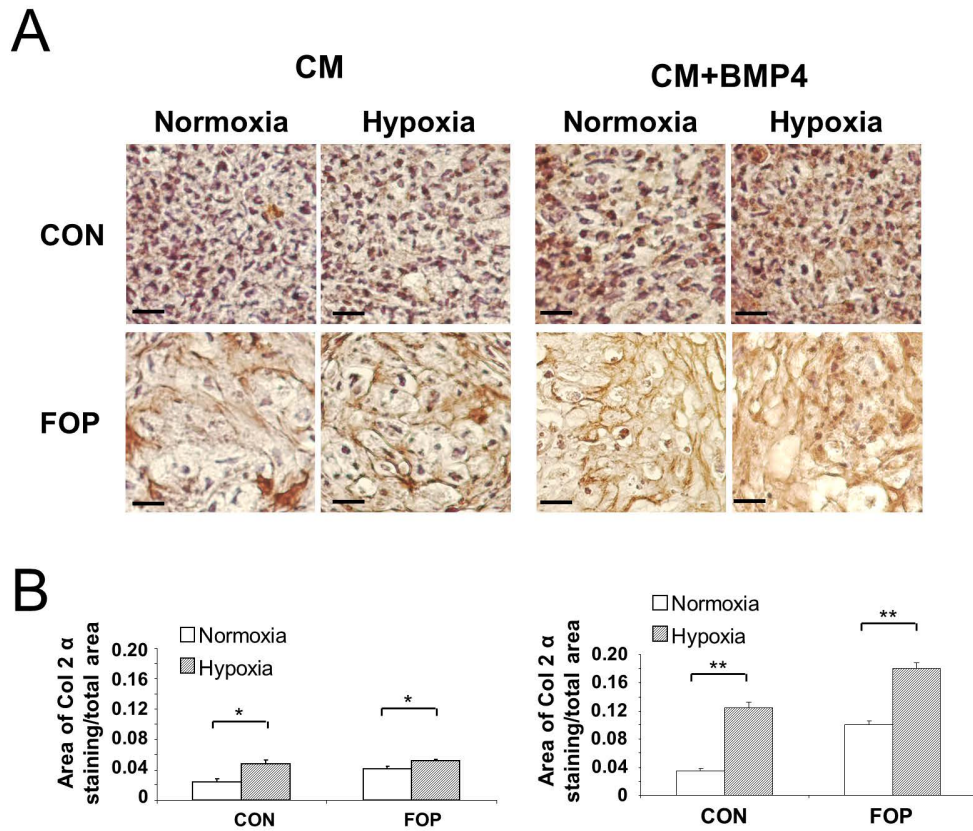
HIF-1 α mimetic CoCl₂ reproduces the effects of hypoxia on ligand-independent BMP signaling in SHED cells. Genotype and hypoxia are significantly related to (a) ID1 mRNA expression and (b) SMAD 1/5/8 phosphorylation by 2-way ANOVA ($p < 0.0001$). Bonferroni post-hoc analysis: ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; $n = 3$. C, control; F, FOP; CoCl₂, cobalt chloride.



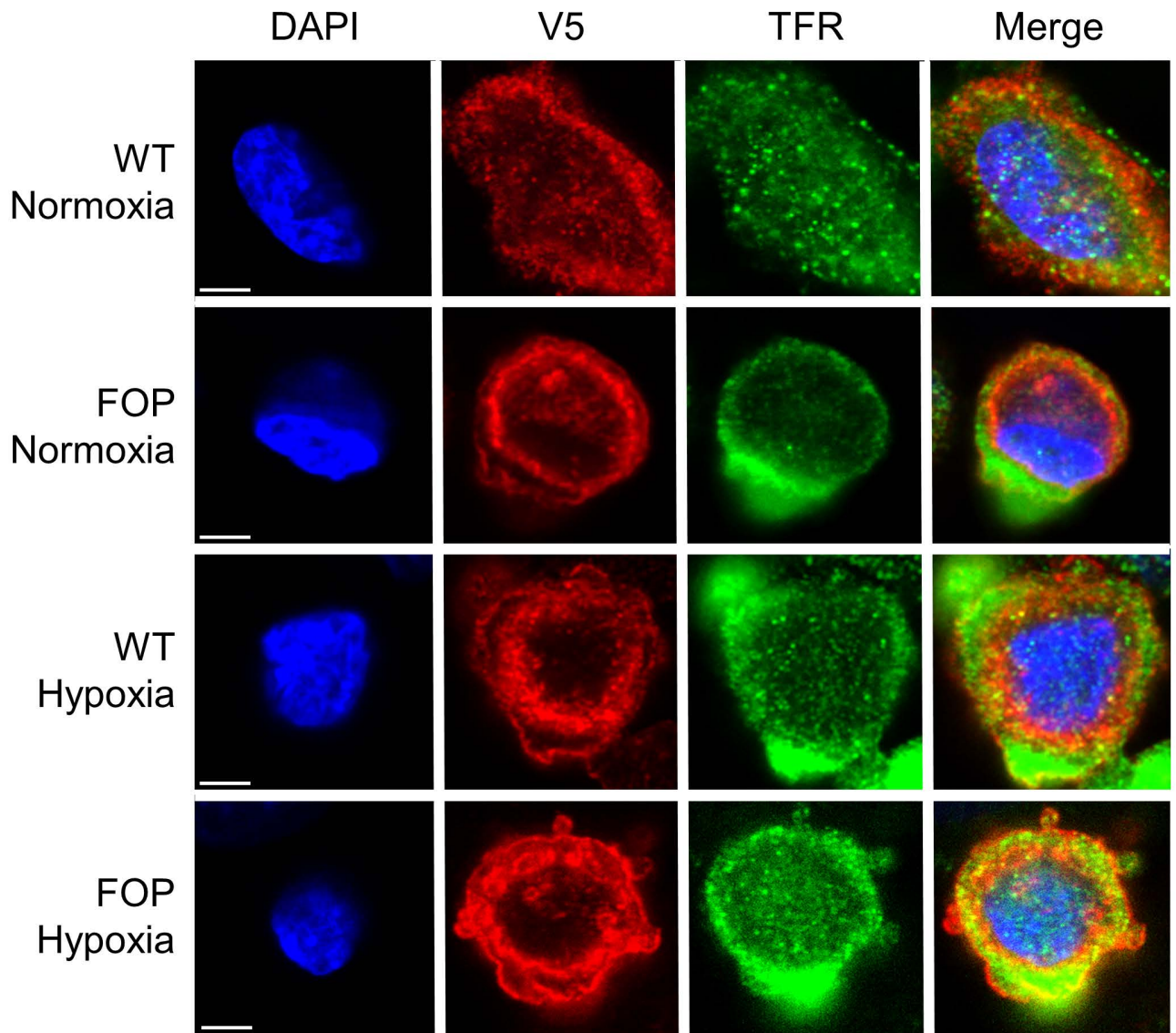
LDN-193189 mitigates SMAD 1/5/8 phosphorylation in SHED cells under low oxygen conditions. SMAD 1/5/8 phosphorylation was detected by Western blot analysis after two hours under hypoxia. Genotype and drug inhibition are significantly related to SMAD 1/5/8 phosphorylation by 2-way ANOVA ($p < 0.01$ and $p < 0.0001$, respectively). Bonferroni post-hoc analysis: ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; $n=2$; C, control; F, FOP.



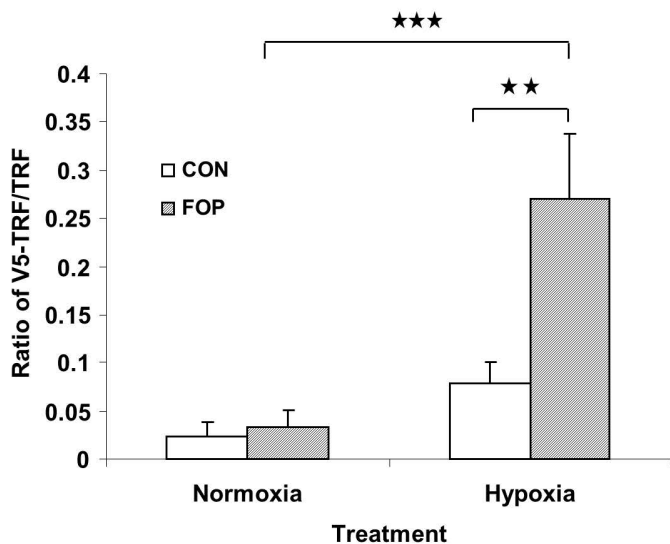
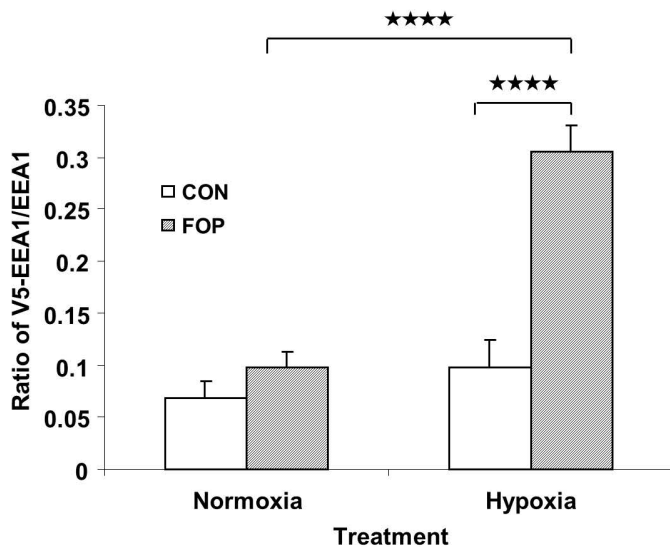
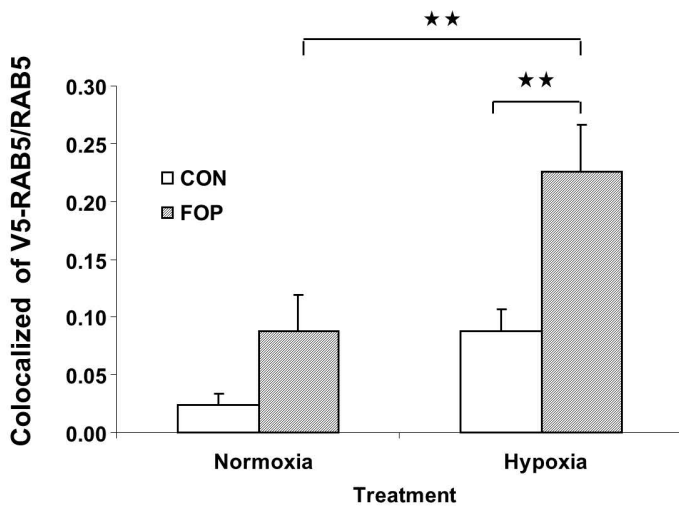
Reduced binding of FKBP12 to mutant ACVR1 is enhanced under hypoxia. Following the indicated treatment under hypoxic conditions, proteins were immunoprecipitated with ACVR1 antibody and then immunoblotted with anti-FKBP12 antibody. p23 is a co-immunoprecipitated protein used as normalization standard. Genotype and hypoxia are significantly related to FKBP12 binding by 2-way ANOVA ($p < 0.001$ and $p < 0.01$, respectively). Bonferroni post-hoc analysis: *, $p < 0.05$; $n = 3$; C, Control; F, FOP.



mACVR1 and BMP enhances chondrogenesis of SHED cells. (A) Histological appearance and (B) quantification of col2 α (n = 3). CM, chondrogenic media; BMP, bone morphogenetic protein. **, p < 0.01; *, p < 0.05. Scale bar = 100 μ m. Note that CM+BMP4 normoxia controls express greater col2 α than those without BMP (p < 0.05).



Hypoxia promotes enhanced endosomal retention of mutant ACVR1. V5-tagged wild-type and mutant ACVR1 is localized to TFR-positive endosomes (n = 9). Scale bar = 10 μ m.



Quantification of endosomal co-localization of V5-labeled wild-type and mutant ACVR1 under normoxia and hypoxia. Endosomal markers are RAB5, EEA1, and TRF. ACVR1 endosomal co-localization is expressed as the ratio of merged fluorescence signal/endosome marker fluorescence signal. Genotype and oxygen tension are significantly related to ACVR1 endosomal co-localization by 2-way ANOVA ($p < 0.05$ and $p < 0.001$, respectively). Bonferroni post-hoc analysis: ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; $n = 9$

FOP

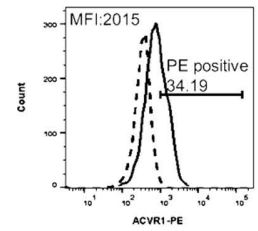
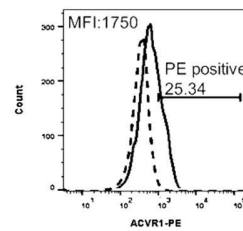
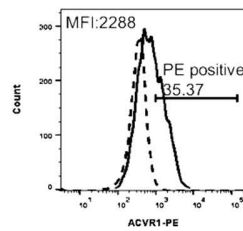
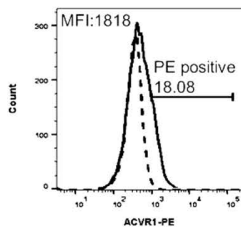
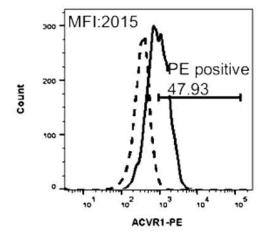
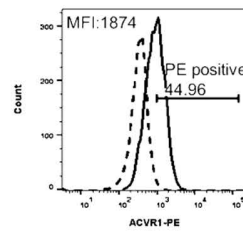
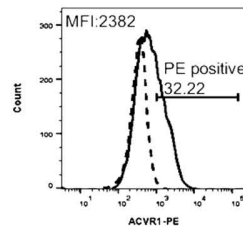
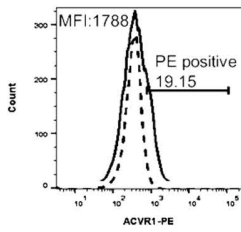
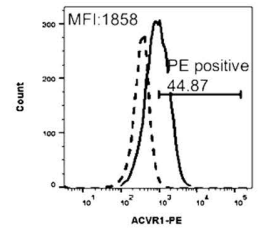
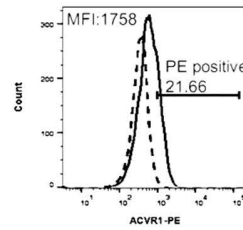
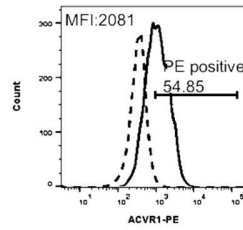
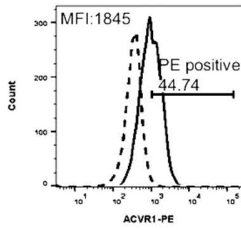
Control

Normoxia

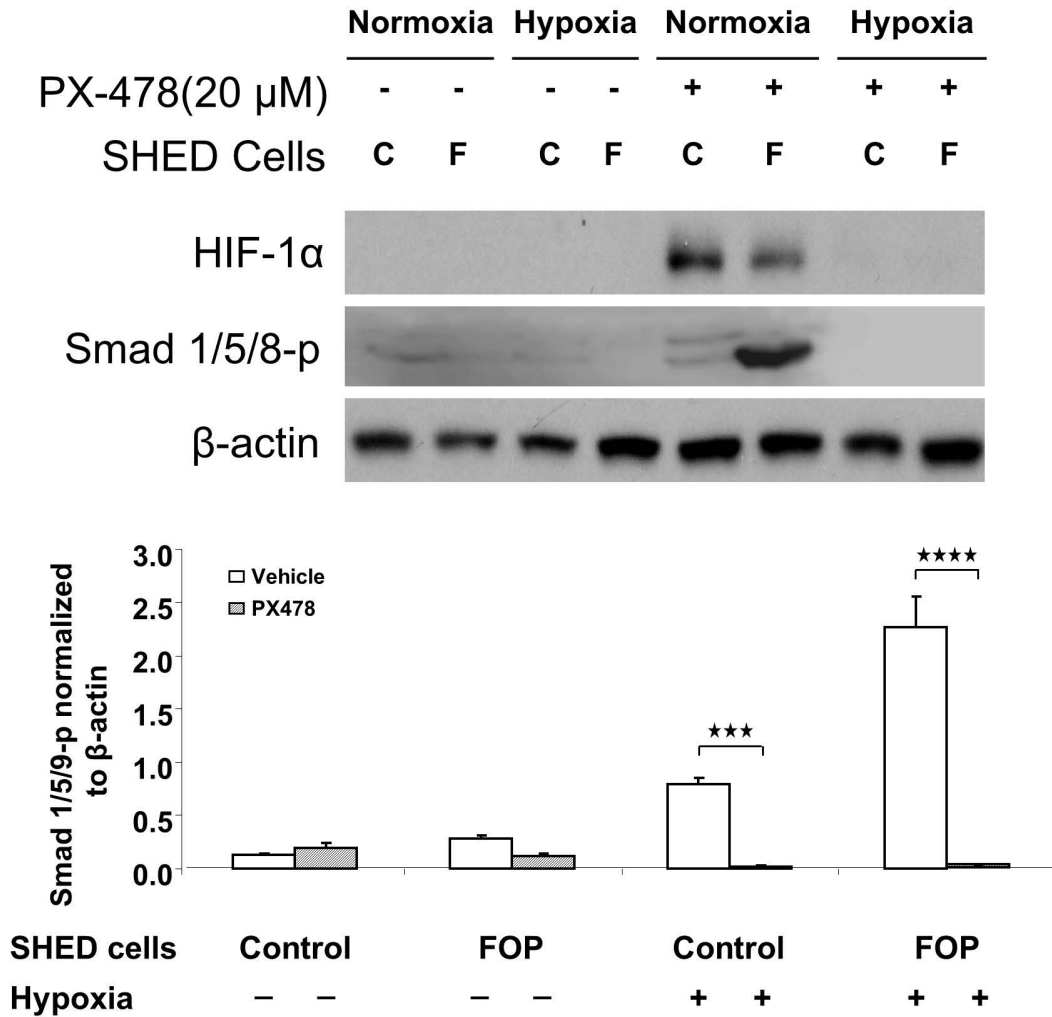
Hypoxia

Normoxia

Hypoxia

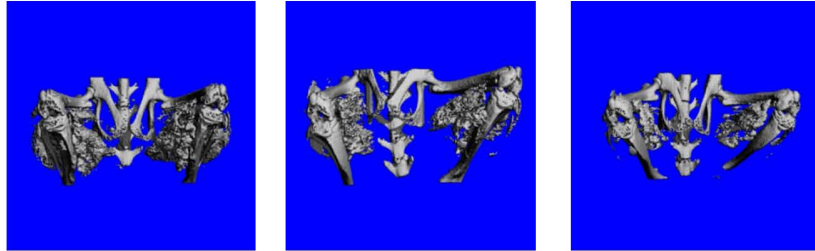
Avg MFI \pm SEM 1818.2 \pm 113.12251.0 \pm 148.81795.0 \pm 115.81963.1 \pm 92.4

Flow analyses are displayed as histograms showing ACVR1-PE fluorescence intensity on the horizontal axis and cell counts on the vertical axis. The unstained negative control is superimposed on the experimental plot (dashed line). The percentage of ACVR1-PE positive cells and the median fluorescence intensity (MFI) are indicated on each plot and the average (avg) MFI for each group is displayed. Shown are representative plots for each of three replicate SHED cell strains cultured for each genotype and fluorescently stained with ACVR1-PE under normoxic or hypoxic conditions ($n = 9$ for each oxygen tension group).

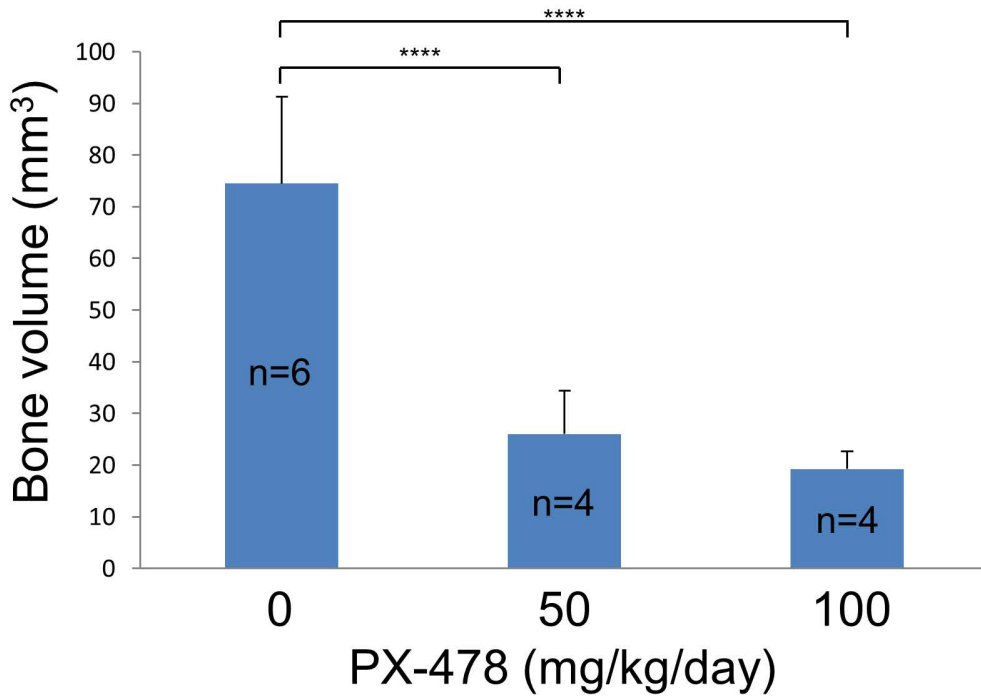


PX478 blocks ligand-independent BMP signaling in vitro. Hypoxia and HIF-1 α inhibition are significantly related to SMAD 1/5/8 phosphorylation by 2-way ANOVA ($p < 0.0001$; interaction, $p < 0.0001$). Bonferroni post-hoc analysis: ****, $p < 0.0001$; ***, $p < 0.001$; $n = 3$. Note that quantification is not presented analogously to the lane order in the blot shown.

A



B



PX-478 effectively reduces HEO in a caACVR1 FOP-like mouse model. (A) Representative μ CT images of control (left panel), PX-478 treated mice at 50 mg/kg/day (middle panel), and 100 mg/kg/day (right panel). (B) PX-478 significantly decreases total HEO volume. ****, $p < 0.0001$.

Wang et al. Supplementary Methods

Cell culture

Stem cells from human exfoliated deciduous teeth (SHED cells) were isolated as previously reported ⁽¹⁾ and determined to be mycoplasma-free at the time of use in experiments. Briefly, the dental pulp was digested with 2 mg/ml type II collagenase for 1 hour (37°C) in Dulbeccos Phosphate Buffered Saline (DPBS) and filtered through a 100 µm cell strainer (BD Falcon, Franklin Lakes, NJ). Cells in the filtrate were recovered by centrifugation (400g, 10 min) and

For experimental treatments, SHED cells were plated at 1×10^4 cells/cm² • • • • • and grown for 1–4 days in a humidified atmosphere of 5% CO₂ at 37° before placed under low oxygen conditions (1% O₂) in hypoxia chambers (Billups-Rothenberg Inc., Del Mar, CA) and/or treated with inhibitors. P38 inhibitor SB203580 (Sigma-Aldrich, St. Louis, MO) and dorsomorphin (Sigma-Aldrich, St. Louis, MO) were used at a concentration of 20µM; LDN-193189 was used at a concentration of 20 nM (Selleckchem, Houston, TX); noggin (R&D Systems, Minneapolis, MN) was used at a concentration of 400ng/ml. Apigenin [Sigma-Aldrich, Saint Louis, MO], imatinib (SelleckBio, Houston, TX), and PX-478 (MedKoo Bioscience, Chapel Hill, NC) were used at the indicated concentrations. For other experiments, 500 µM CoCl₂ (Sigma, St. Louis, MO) was added to SHED cells after two hours of serum-deprivation under normoxic conditions.

RNA isolation and real-time PCR

Cells were washed with PBS, and RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA), following the manufacturer's instructions. cDNA was synthesized with the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen/Life Technologies, Grand Island, NY). Real-time PCR reactions contained forward and reverse primers, cDNA (1:5 dilution), and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) as previously described.⁽²⁾ Each sample was analyzed in triplicate (Applied Biosystems 7500 Sequence Detection System), and target gene

• • • • •

RNA. The following primer sequences were used:

[RABAPTIN 1] 5'-CGCAGAGAGGGAAATAGCTG-3' (forward)

5'-CTTGGGCCTTTTTTCATTTCA-3' (reverse)

[ID1] 5'-AGTGGTGCTTGGTCTGTCG-3' (forward)

5'-GCTCCTTGAGGCGTGAGTAG-3' (reverse)

[18S rRNA] 5'-ATCCCTGAAAAGTTCCAGCA-3'(forward)

5'-CTGCTTTCCTCAACACCACA-3'(reverse)

[ACVR1] 5'-CTGCGGTAATGAGGACCACTGT-3'(forward)

5'-CATAAACCTGGAAGCAGCCTTT-3' (reverse)

[VEGF] 5'-GTTCATGGATGTCTATCAGCGCAGCTACTG-3' (forward)

5'-GGGGCACACAGGATGGCTTGAAGATGTACT-3' (reverse)

[PGK-1] 5'-GCAGATTGTGTGGAATGGTC-3' (forward)

5'-CCCTAGAAGTGGCTTTCACC-3' (reverse)

[COL2A1] 5'-GACAATCTGGCTCCCAAC-3' (forward)

5'-ACAGTCTTGCCCCACTTAC-3' (reverse)

[Sox 9] 5'-CTTTGGTTTGTGTTTCGTGTTTTG-3' (forward)

5'-AGAGAAAGAAAAAGGGAAAGGTAAGTTT-3' (reverse)

[ACAN] 5'-GTCTCACTGCCCAACTAC-3' (forward)

5'-GGAACACGATGCCTTTCAC-3' (reverse)

[MSX2] 5'- CGGTCAAGTCGGAAAATTCA-3 (forward)

5'- GCTTCCGATTGGTCTTGTGT-3 (reverse)

Western blot analysis

Cells were lysed in RIPA buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) [Sigma-Aldrich, St. Louis, MO] with 1× proteinase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL). The lysate was incubated on ice for 15 min and centrifuged at 12,000 g for 10 minutes. The amount of protein in the supernatant was quantified using the BCA Protein Assay kit (Thermo Scientific, Rockford, IL). 50 µg of total protein were separated on a 10% SDS-PAGE gel and then transferred to Nitrocellulose membranes (Invitrogen, Grand Island, NY) by electroblotting. Membranes were incubated overnight at 4°C with a 1:1000 dilution of antibody specific for phosphoSmad1/5/8 (#9511S; Cell Signaling, Danvers MA), SMAD 1/5 (#ab75273; ABCAM, Cambridge, MA), HIF1-• (#NB100-479; Novus Biologicals, Littleton, CO), Rabaptin 5 (#610676; BD Biosciences, San Jose, CA, Tie2 (#ab24859; ABCAM, Cambridge, MA, or -actin (#SC47778; Santa Cruz Biotechnology, Santa Cruz CA) in PBS containing 5% nonfat milk (except for detection of phosphoSmad1/5/8, where 5% BSA was used). Membranes were washed with TBST (PBS + 0.1% Tween-20) and incubated for 1 hour with the species-appropriate secondary antibody conjugated with horseradish peroxidase (#SC2768, #SC2005; Santa Cruz Biotechnology, Santa Cruz CA) at a final concentration of 0.1 µg/ml. An Immobilon chemiluminescent horse radish peroxidase substrate Western blotting detection system (Milipore, Billerica, MA) was used to detect the antigen-antibody complex. Protein bands were quantified using Image J software (<http://imagej.nih.gov/ij/>).

albumin (BSA) in PBS for 30 minutes. Primary antibody against HIF- α (1:479; Novus Biological, Littleton, CO) was diluted 1:600 based on the manufacturer's recommendation and experience from previous use, and incubated with cells in a humidified container for 2 hours. Unbound primary antibody was washed out with three rinses of PBS before incubation of cells with fluorescently-tagged Alexa 488 secondary antibody (#A11034; Invitrogen Grand Island, NY). DAPI was added at 0.2 $\mu\text{g}/\text{ml}$ for 5 minutes. After rinsing cells three times with PBS, cover slips were mounted onto slides. All steps were performed at room temperature. Preimmune serum was used as a negative control at a dilution comparable to that of the primary antibody. All dilutions were made in blocking solution. Slides were stored at 4°C in the dark. Cells were visualized using a Nikon Eclipse 90i fluorescence microscope and Nikon Plan Fluor 10X0.30 and 20X0.45 objectives. Image capture was performed using NIS Elements Imaging Software 3.10 Sp2 and a Photometrics Coolsnap EZ camera. Emission times were standardized to negative controls.

Detection of V5, EEA1, RAB, and transferrin receptor (TFR) in C2C12 cells

C2C12 cells were seeded in multiwell chamber plates at 5×10^3 cells/cm² in culture medium without antibiotics. After 24 hours, wild-type or ACVR1 (R206H) expression vectors tagged with V5 were constructed as previously described⁽³⁾ and transfected into cells using lipofectamine 2000 (Invitrogen Grand Island, NY) according to the manufacturer's protocol. Two days after transfection, cells were serum-starved for two hours and then placed under hypoxic conditions (1% pO₂) for 2 hours. Cells were washed with PBS, fixed with 4% paraformaldehyde, and were permeabilized with 0.1% Triton X-100 for 5 minutes. Fixed cells

were incubated with blocking solution (4% BSA in 1X PBS) (1 hour at room temperature) and then with primary antibodies against V5 (#R960-25; Invitrogen Grand Island, NY), EEA1 (#ab50313, ABCAM, Cambridge, MA; #610457, BD Bioscience, San Jose, CA), RAB5 (#R4654, Sigma-Aldrich, St. Louis, MO; #ab18211, ABCAM, Cambridge, MA), or TFR (#ab84036; ABCAM, Cambridge, MA), each at a final concentration of 5µg/ml (18 hours at 4 °C). Alexa 555-tagged secondary antibody against mouse anti-human V5 (#A21425; Invitrogen Grand Island, NY) and Alexa 488-tagged secondary antibodies (#A11034 Invitrogen Grand Island, NY) against rabbit anti-human EEA1, rabbit anti-human RAB5, and rabbit anti-human TFR were each used at a final concentration of 1 µg/ml (2 hours at room temperature). DAPI staining (0.2 µg/ml; 5 minutes at room temperature) and PBS washes (3 × 5 minutes each) were performed before image capture immunofluorescence microscopy as described above.

Quantification of ACVR1 endosomal co-localization

Merged images showing V5-labeled wild-type or mACVR1 and an endosomal marker (EEA1, RAB5, TFR) were quantified for overlapping signal by automated counting of pixels in the yellow color range using the histogram feature on the color range menu in Adobe Photoshop CS5. Similarly, images showing endosomal markers were quantified using the same histogram feature and automated counting of pixels in the green color range. ACVR1 endosomal co-localization was expressed as the ratio of merged fluorescence signal area (number of yellow pixels)/endosome marker fluorescence signal area (number of green pixels).

Localization of hypoxic tissue

Tissues were harvested on days 0, 2, and day 7 from caACVR1 (Q207D) mice after treatment with Adenovirus-Cre and cardiotoxin. Twenty-four hours before harvesting, mice were injected (intraperitoneal) with 10 mM EF5, a pentafluorinated derivative of etanidazole at 1% of body weight. Hypoxic cells can be identified using 2-nitroimidazole drugs such as pimonidazole and EF5, which bind protein and DNA at oxygen concentrations less than two percent.⁽⁵⁾Tissues were fixed at 4% PFA, decalcified using Immunocal (Decal Chemical Corporation, Tallman, NY), embedded in paraffin, and sectioned serially at 7 μ m, and stained with hematoxylin and eosin (H&E) by standard procedures. EF5 immunostaining was performed using a Cy3-conjugated antibody as previously described.⁽⁶⁾

Detection of HIF-1 • • • • • -hereditary HO

Tissue sections were subjected to antigen retrieval (95°C x 20 minutes in 10 mM sodium citrate buffer, pH 6.0). The sections were permeabilized by 0.1% Triton X-100 for 5 minutes at room temperature and incubated with blocking solution (10% normal goat serum) for 1 hour at room • • • • • -479;
Novus Biologicals, Littleton, CO) at 5 μ g /ml for 18 hours at 4 °C, followed by incubation of secondary Alexa 488 goat anti-mouse antibody (1 μ g/ml; 2 hours 4 °C) (#A11029; Invitrogen/Life Technologies, Grand Island, NY). DAPI staining (0.2 μ g/ml; 5 minutes at room temperature) and PBS washes (3 \times 5 minutes each) were performed before image capture immunofluorescence microscopy as described above. In FOP sections, at least 50 cells per 200X field and at least 500 total cells were scored per cell type.

Flow Cytometry

Cell preparation

Control and FOP SHED cells were cultured for 16 hours under hypoxic and normoxic conditions and then immunolabeled under the same conditions. Cells were harvested using Cellstripper non-enzymatic cell dissociation solution (Mediatech, Herndon, VA) to avoid compromising surface antigens. Labeled cells were then washed with and resuspended in 1X FACS buffer at a concentration of 3×10^5 cells/ml for flow cytometric analysis.

Immunofluorescence labeling

SHED cells were stained with anti-ACVR1-phycoerythrin (PE) at 200 $\mu\text{g/ml}$ (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and Live/Dead Aqua (LDA) reagent (Fixable Cell Stain Kit, Invitrogen, Carlsbad, CA). Optimal working concentration for anti-ACVR1-PE was determined by titration to be 24 μl per $2-10 \times 10^5$ cells. A standard staining protocol for surface molecules was followed;⁽⁷⁾ however, cells maintained in culture under hypoxic conditions were stained under hypoxic conditions. Cells were blocked with 10 μl of 1 mg/ml Human type AB serum (Fisher Bioreagents, Pittsburgh, PA) at room temperature for 10 minutes, and then incubated with anti-ACVR1-PE at 4 °C for 30 minutes in the dark. CompBeads (BD Biosciences, San Jose, CA) stained with ACVR1 were incubated under the same conditions according to the manufacturer's instructions (http://wwwbdbiosciences.com/documents/BD_FACSDiva_setup_system.pdf). Unstained SHED cells and CompBeads were used as negative controls. All samples were then washed twice in FACS buffer. After washing, 1 μl of LDA reagent was

added to each sample and incubated at 4 °C for 30 minutes in the dark. Samples were washed once with FACS buffer and then fixed with 400 ul of 4% paraformaldehyde for 15 minutes at 4 °C in the dark. All samples were then resuspended in 400 µl FACS buffer for flow analysis.

Analysis

For each of three FOP and three control SHED cell strains, triplicate cultures were harvested and stained under hypoxia and normoxia. 10,000 total events for each sample were analyzed using a Becton Dickinson FACS Canto A instrument running DiVa software (BD, Franklin Lakes, NJ). Unstained or singly stained CompBeads were used to set compensation controls for the instrument in PE and LDA reagent channels using FlowJo analytical software (Treestar, Ashland, OR) and according to the manufacturer's instructions (<http://www.flowjo.com/v765/en/overview.html>). The compensation controls were then used to set the photomultiplier tube (PMT) values for the fluorophores.

FlowJo analytical software was used for offline analysis. The gating strategy was as follows: Negative staining by the Live/Dead Aqua reagent was used to gate viability. Doublet populations were removed by gating single cells in both forward scatter (FSC) and Side scatter (SSC) channels. Positive ACVR1-PE cell populations were gated using the unstained control cells. Median fluorescent intensity (MFI) of ACVR1-PE in SHED cells was evaluated for all samples by Flowjo software and average MFI was determined for each experimental group.

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