

INHIBITION OF THE OXYGEN SENSOR PHD2 ENHANCES TISSUE-ENGINEERED ENDOCHONDRAL BONE FORMATION

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The authors have nothing to disclose

ABSTRACT

Tissue engineering holds great promise for bone regenerative medicine, but clinical translation remains challenging. An important factor is the low cell survival after implantation, primarily caused by the lack of functional vasculature at the bone defect. Interestingly, bone development and repair initiate predominantly via an avascular cartilage template, indicating that chondrocytes are adapted to limited vascularization. Given these advantageous properties of chondrocytes, we questioned whether tissue-engineered cartilage intermediates implanted ectopically in mice are able to form bone, even when the volume size increases. Here, we demonstrate that endochondral ossification proceeds efficiently when implant size is limited ($\leq 30 \text{ mm}^3$), but chondrogenesis and matrix synthesis are impaired in the center of larger implants, leading to a fibrotic core. Increasing the level of angiogenic growth factors does not improve this outcome, because this strategy enhances peripheral bone formation, but disrupts the conversion of cartilage into bone in the center, resulting in a fibrotic core, even in small implants. On the other hand, activation of hypoxia signaling in cells before implantation stimulates chondrogenesis and matrix production, which culminates in enhanced bone formation throughout the entire implant. Together, our results show that induction of angiogenesis alone may lead to adverse effects during endochondral bone repair, whereas activation of hypoxia signaling represents a superior therapeutic strategy to improve endochondral bone regeneration in large tissue-engineered implants.

KEYWORDS

Bioengineering, Implants, Chondrocyte and cartilage biology, Paracrine pathways, Animal models

1. INTRODUCTION

Cell-based tissue engineering is a promising approach to treat non-healing bone defects, but translation into clinical therapies remains limited because survival of the implanted cells is often poor. Already in the first few days, up to 90% of the implanted cells undergo cell death ^(1,2,3). This outcome is likely caused by the lack of functional blood vessels in the injured tissue, leading to a nutrient-depleted and hypoxic environment. Host-driven revascularization of the implanted tissue mitigates this problem, but it is a slow process (less than 1 mm / day ⁽⁴⁾) and therefore insufficient to timely prevent the lack of nutrients and oxygen, especially in larger tissue defects.

Bone tissue-engineered implants consist usually of a scaffold seeded with cells and/or growth factors. The scaffold is generally a natural or synthetic polymer, such as collagen or poly-lactic acid, or a ceramic such as hydroxyapatite ^(5,6). Mesenchymal stromal cells (MSC, also called mesenchymal stem cells) are used for bone regenerative applications and they are most often isolated from bone marrow, periosteum, or adipose tissue. ⁽⁷⁾. The bone marrow is the most common source for skeletal MSC in the clinical setting, but recent data show that periosteal cells contribute much more extensively to bone repair in small animal models ⁽⁸⁾. Despite the debate on the cell type to be used, the poor survival of cells in the tissue defect is a general phenomenon, necessitating additional strategies to improve the regenerative potential of these implants.

Most traumata of long bones heal via endochondral ossification, characterized by the formation of an initial avascular cartilage template that is subsequently converted into vascularized bone tissue ⁽⁹⁾. Hence, chondrocytes inside this cartilage callus are adapted to a poorly vascularized environment. These beneficial properties of chondrocytes explain why tissue engineering via

the endochondral pathway has become increasingly attractive compared to direct bone formation ^(10,11,12), especially for treating very large skeletal defects. However, developmental studies show that also chondrocytes become hypoxic when the growth plate enlarges ⁽¹³⁾. At that moment, chondrocytes partially rely for their survival on the timely invasion of blood vessels ⁽¹⁴⁾. These data suggest that the dimensions of an avascular chondrogenically engineered tissue may be confined, and this size limitation may hamper translation to successful treatment of clinically relevant defects.

Possible strategies to promote cell survival in engineered tissues comprise stimulation of angiogenesis or activation of the hypoxia signaling pathway. Blood vessels are indeed essential for tissue regeneration in general and for bone repair in particular, because osteogenesis is strongly linked to angiogenesis ⁽¹⁵⁾. Stimulation of vascularization ⁽¹⁶⁾ by supplementation of angiogenic growth factors, like vascular endothelial growth factor (VEGF) ⁽¹⁷⁾, can enhance direct, also called intramembranous, bone repair ^(15,18). However, it is not known whether this strategy also improves tissue-engineered bone formation via the endochondral pathway. The conversion of cartilage into bone is associated with vascular invasion ^(15,19), but findings also suggest a rather disadvantageous effect of VEGF on chondrocytic properties *per se*. Indeed, *in vivo* and *in vitro* chondrogenesis and cartilage formation by MSC is promoted when VEGF signaling is inhibited ⁽²⁰⁾. It remains therefore crucial to determine whether stimulating angiogenesis improves the regenerative outcome of cell-based implants that form bone through a cartilage intermediate.

A second strategy to increase the survival of the implanted cells is to use hypoxic preconditioning by activating the hypoxia signaling pathway. When sufficient oxygen is available, the hypoxia inducible factor (HIF)-1 α is targeted for proteasomal degradation after

hydroxylation by prolyl hydroxylase domain-containing proteins (PHD) ⁽²¹⁾. On the other hand, when oxygen tension decreases, HIF-1 α accumulates and exerts its transcriptional activities. The HIF pathway activates not only pro-angiogenic signaling, but also adaptations in cellular metabolism, redox homeostasis and biosynthetic processes ⁽²²⁾. As a result, cells become better adapted to hypoxic stress, as has also been observed in bone development and regeneration. During bone development, hypoxic chondrocytes rely on HIF-1 α signaling for their survival and function ^(14,23,24). In bone tissue-engineered implants, activation of hypoxia signaling enhances direct bone formation ^(3,25). Interestingly, in both settings these effects were only partially mediated via angiogenesis. Activation of hypoxia signaling may thus provide an additional strategy to enhance cell survival and bone regeneration in large tissue-engineered implants ⁽¹²⁾, but whether this is also applicable to the endochondral bone repair process is not known.

In this study, we investigated whether increasing the size of an engineered endochondral cartilage intermediate limits the survival of the embedded chondrocytes when implanted ectopically in mice. In addition, we questioned whether supplementation of VEGF or hypoxic preconditioning improves the regenerative outcome. We demonstrate that chondrocytes are well-adapted in small implants, but chondrogenesis fails when the size of the implant increases. Supplementation of VEGF stimulates angiogenesis but also disrupts the conversion of immature cartilage into bone, leading to a poor regenerative outcome of the endochondral intermediate. In contrast, activation of hypoxia signaling enhances chondrogenesis and bone formation in large implants, indicating that this strategy holds therapeutic potential for endochondral bone tissue engineering.

2. MATERIALS & METHODS

2.1 Animals

Wild type C57Bl/6 mice (R. Janvier Breeding Center, Saint-Berthevin, France), mice constitutively expressing enhanced green fluorescent protein (eGFP; Tg(CAG-EGFP)) or mTomato (Gt(ROSA)26Sor^{tm4}(ACTB-tdTomato,-EGFP)^{Luo}), and mice expressing dsRed under the control of the collagen type I (Col1a1) promoter (Tg(Col1a1-cre/ERT2,-dsRed)^{1Smkm}) were used at 8 weeks of age. *Phd2^{fl/fl}* mice were used for genetic activation of the hypoxia signaling pathway ^(3,26). Since previous bone-healing studies from our lab did not find sex-specific differences, we here used female mice in all experiments ^(3,27,28,29). Animals were kept in non-SPF housing at 22°C under a 14h light / 10h dark cycle and fed normal chow diet (V1535, Ssniff GmbH, Soest, Germany) *ad libitum*. All animal experiments were conducted according to the regulations and with approval of the Animal Ethics Committee of the KU Leuven.

2.2 Cell cultures

Murine periosteum-derived cells (mPDC) were obtained as described previously ⁽²⁷⁾. Briefly, tibiae and femora were dissected and cleaned, their epiphyses were coated in 5% low melting point agarose (SeaPlaque, Lonza, Verviers, Belgium) and stromal cells of the periosteal surface were released by sequential collagenase-dispase digests. The cell suspension was washed, seeded and cultured in α MEM medium supplemented with 2 mM GlutaMAX-I, 10% fetal bovine serum (FBS), 100 U/ml penicillin and 50 μ g/ml streptomycin (all from Gibco, Life Technologies, Ghent, Belgium), further referred to as growth medium.

To obtain murine bone marrow stromal cells (mBMSC), bone marrow was flushed from tibiae and femora using growth medium as previously described ⁽²⁸⁾. The non-adherent cells were removed 48 hours after seeding by washing with phosphate-buffered saline (PBS, Gibco), and the adherent cells were cultured further until subconfluence. Prior to experiments, mBMSC

were negatively selected for CD45 via MACS separation using CD45 MicroBeads and an LS column (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions.

To obtain chondrogenic differentiation after implantation, mPDC or mBMSC were pretreated during *in vitro* culture with 5 ng/ml human recombinant fibroblast growth factor (FGF) 2 (mPDC^{FGF2}; R&D Systems, Minneapolis, MN, USA) and 5 U/ml heparin (LEO Pharma, Ballerup, Denmark), as described previously ⁽²⁸⁾.

To analyze *in vivo* the differentiation of donor cells into osteoblasts, the lentiviral Col1a1-GFP reporter (see Molecular Techniques) was transduced into mPDC^{FGF2} at multiplicity of infection (MOI) 50 in the presence of 8 µg/ml polybrene (Sigma-Aldrich, Diegem, Belgium). After 24 hours, the medium was refreshed, and another 48 hours later, the cells were used for further experiments.

To obtain VEGF overexpression, mPDC were incubated with 50 plaque-forming units of adenovirus expressing VEGF164 (Gene Transfer Vector Core, University of Iowa, IA, USA) or GFP as a control.

To delete PHD2, mPDC isolated from *Phd2^{fl/fl}* mice were treated with adenovirus expressing Cre, or GFP as control, at a MOI of 500. After 24 hours, the medium was refreshed, and another 72 hours later, the cells were used for further experiments. For pharmacological inhibition of PHDs, mPDC^{FGF2} were treated with IOX2 (Sigma-Aldrich) at 10 µM for 72 hours prior to implantation. DMSO (Sigma-Aldrich) was used as vehicle-control at the same dilution. A summary of the different cell types used is provided in table S1.

2.3 *In vitro* differentiation assays

For osteogenic differentiation, mPDC^{FGF2} were seeded in growth medium at a density of 3 * 10⁴ per cm², and upon confluence, the medium was switched to growth medium supplemented

with 10 mM β -glycerophosphate and 50 μ g/ml ascorbic acid (Sigma-Aldrich). After 14 days, cultures were stained for alkaline phosphatase or with Alizarin Red using routine protocols ⁽²⁷⁾, or cells were used for RNA extraction.

Chondrogenic differentiation was assessed using micromass culture, where 2×10^5 mPDC^{FGF2} or mBMSC^{FGF2} were seeded in a 10 μ l droplet of growth medium and left to adhere for 2 hours. Subsequently, growth medium supplemented with 10 ng/ml transforming growth factor (TGF)- β 1 (PeproTech, Rocky Hill, CT, USA), 20 μ M Rho-kinase inhibitor (Y27632; Axon Medchem, Groningen, The Netherlands) and 50 μ g/ml ascorbic acid was added. After 9 days, micromasses were stained with Alcian Blue 8GX (1% in 0.1 N HCl; pH 1.2; Sigma-Aldrich) or used for RNA extraction. Alcian Blue staining intensity was quantified by measuring absorbance at 620 nm after dye extraction with guanidine hydrochloride (6 M; Thermo Fisher Scientific, Asse, Belgium) for 6 hours.

2.4 Molecular techniques

To detect collagen type I expressing donor-derived cells, a Colla1-GFP reporter was made. To this end, the 3.2kb Colla1 promoter fragment was released from pJ320 (a kind gift from S. Murakami) using XhoI and Sall. This fragment was then cloned into the pWPXLd plasmid (#12258; Addgene, Cambridge, MA, USA), from which the EF1 promoter was removed using Sall and BamHI.

Measurement of DNA was performed by lysing cells in a homogenization buffer (50 mM Na₂HPO₄, 2 M NaCl and 2 mM EDTA; pH 7.4), whereafter DNA content was measured using Hoechst 33342 (Calbiochem, San Diego, CA, USA). VEGF concentrations were measured in culture medium using the Mouse VEGF DuoSet ELISA kit (R&D Systems) according to the manufacturer's instructions and they were normalized to DNA. Western blots for PHD2 and

HIF-1 α were performed as described before ^(3,27). Briefly, cultured cells were incubated with lysis buffer containing 10 mM KCl, 20mM HEPES (pH 7.9), 1.5 mM MgCl₂, 1 mM EDTA, 0.5% NP40 and 1 mM DTT and supplemented with phosphatase inhibitors (1 mM Na₃VO₄, 20 mM NaF) and protease inhibitors (1 mM PMSF, 5 μ g/ml aprotinine, 5 μ g/ml leupeptin, 0.33 μ g/ml antipain). 20 μ g of protein extract was loaded in 20 μ l on SDS PAGE.

2.5 Quantitative Real-Time PCR

Total RNA was extracted from cells using the NucleoSpin RNA kit (Macherey-Nagel GmbH, Düren, Germany) according to the manufacturer's instructions. RNA (1 μ g) was reverse transcribed into cDNA using SuperScript II Reverse Transcriptase (Invitrogen, Thermo Fisher Scientific) and used at a concentration of 1% (v/v) in subsequent qRT-PCR reactions. qRT-PCR was performed using a StepOnePlus Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific). Gene-specific forward and reverse primers as well as probes with fluorescent dye (FAM) and quencher (TAMRA) were used (for sequences see table S2). Expression levels were analyzed using the $2^{-\Delta\Delta C_t}$ method and were normalized for the expression of the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (*Hprt*).

2.6 Scaffold implantations

mPDC^{FGF2} (passage 3) or mBMSC^{FGF2} (passage 2) were embedded in collagen type I (5 mg/ml in PBS; Corning GmbH, Wiesbaden, Germany) at a density of 1×10^7 cells per ml, and 100 μ l was implanted subcutaneously as previously described ⁽²⁸⁾. To generate larger implants, 300 μ l of the collagen-cell suspension was gelled at 37°C in agarose molds for 20 minutes, after which they were kept on ice in Hanks' Balanced Salt Solution (Gibco, Life Technologies) for maximally 30 minutes and implanted subcutaneously via an incision. For pharmacological inhibition of PHDs *in vivo*, IOX2 (10 μ M) or vehicle was injected locally around the implant

every other day. To visualize hypoxic regions in implants mice were injected with pimonidazole (Natural Pharmacia International, Burlington, MA, USA) 180 minutes prior to sacrifice according to the manufacturer's instructions.

The bone-forming capacity of mPDC^{FGF2} at an orthotopic site was assessed using an in-house developed non-healing bone defect model in the mouse tibia ^(3, 28). Briefly, a 4 mm mid-diaphyseal segment was excised from the tibia using a diamond saw disk. Into this defect a 2.5 x 2.5 x 5 mm NuOss calcium/phosphate-collagen scaffold (ACE Surgical, Brockton, MA, USA) was inserted, which was seeded with 1×10^6 mPDC^{FGF2} (passage 3) seeded. The defect was then stabilized using a custom-made external fixator, and samples were retrieved 8 weeks after implantation.

2.7 Intravital imaging

Intravital imaging was performed as described before ⁽²⁹⁾. Briefly, mPDC^{FGF2} seeded in collagen type I were implanted under a custom-made imaging window inserted in the skin. *In vivo* imaging on anesthetized mice was performed using a TCS SP8-MP microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a HCX IRAPO L 25x/0.95 water immersion objective and a Chameleon Ultra II Ti:Sapphire laser (Coherent Inc. Santa Clara, CA, USA) tuned to 920 nm. At this wavelength, eGFP and dsRed undergo excitation and collagen second-harmonic generation (SHG) occurs at 460 nm. For visualization of the host vasculature, 100 µl of 10 mg/ml FITC-dextran (70 kDa; Sigma-Aldrich, Diegem, Belgium) in 0,9% NaCl was injected via the tail vein just before the experiment. Acquired image stacks were merged via maximal intensity projection using ImageJ v1.51 ⁽³⁰⁾ or LAS AF 3.2 software (Leica Microsystems). Images were aligned using second-harmonics generation from extracellular matrix with custom-made MeVisLab software (MeVis Medical Solutions AG, Bremen, Germany). Migration was quantified by measuring the movement of the point

equidistant between the leading and trailing edge of each cell in the X-Y plane. A cell was considered actively migrating when its displacement exceeded twice the average alignment error of the matrix between every measured time point and the next, across all time points. This minimal displacement corresponded to a median total distance of 33 μm (range: 26 – 53 μm) over the entire experiment. For each implant, between 16 and 24 actively migrating cells were analyzed over time. Movement was also measured in the X-Z plane, but was found to be minimal because the total distance covered by each cell increased by less than 4 μm in all cases (*e.g.* a cell moving 186 μm in the horizontal plane over the course of the experiment also moved 13 μm in the vertical plane, resulting in a total movement of 188 μm).

2.8 Immunohistochemistry

Following ectopic implantation, constructs were recovered and fixed in 2% paraformaldehyde at 4°C overnight. Samples were decalcified in EDTA for 14 days at 4°C, embedded in NEG-50 frozen section medium (Richard-Allen Scientific, Thermo Fisher Scientific) and sectioned with a cryostat at 7 μm . Sections were stained with haematoxylin & eosin (H&E), counterstained with silver nitrate to visualize reticulin, Toluidine Blue, or immunostained for sex-determining region Y box 9 (SOX9), collagen type II, phosphorylated histone H3 (H3-p), bromodeoxyuridine (BrdU), or CD31, and DNA was counterstained with Hoechst 33342 using routine protocols published previously^(28,31,32,33). Pimonidazole adducts were detected using the Hypoxyprobe-1 PLUS kit (Natural Pharmacia International). Primary antibodies used are summarized in table S3. Negative controls for immunostainings consisted of absence of primary antibody and are shown in Figure S1. Images were taken on an Axioplan 2 light microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) using a Plan-Neofluar 20x/0.5 objective and processed with ImageJ v1.51 software. Quantification was performed by counting all cells in at least 6 microscopic fields, of 670 μm by 530 μm each, per section, and at least 3 sections per

sample were counted. The periphery was considered the region starting from the edge of the implant to 1 mm inside. The rest was considered center.

2.9 Micro-computed tomography (μ CT)

Following sample retrieval and fixation, implants were scanned in distilled water using a SkyScan 1172 high resolution μ CT (Bruker microCT, Kontich, Belgium) at a voxel size of 5 μ m with a tube voltage of 60 kV, a 0.5 mm aluminum filter, and an integration time of 590 ms. Reconstruction was performed with NRecon v1.6.8 software and mineralized tissue was quantified using a threshold value of 75 with CTAn v1.13 software (both from Bruker microCT) or ImageJ v1.51.

2.10 Statistical analysis

Unless indicated otherwise, data are presented as mean $\pm$ SEM. The number of biological replicates for each experiment is indicated in the figure legends. Researchers were blinded during data collection and analysis of μ CT, histological, and biochemical assays. Data were analyzed by two-sided Student's *t* test using NCSS statistical software (NCSS LLC, Kaysville, UT, USA). Differences between groups were considered statistically significant at $P < 0.05$.

3. RESULTS

3.1 Characterization of early processes during endochondral bone regeneration

To determine the behavior of implanted skeletal progenitor cells during the first days of endochondral bone regeneration, we used a validated model of *in vivo* endochondral bone formation⁽²⁸⁾. eGFP-expressing (GFP⁺) mPDC were cultured in the presence of FGF2 (mPDC^{FGF2}), seeded in a collagen gel and implanted ectopically. Three days later, implants were recovered and histological analysis confirmed that the implanted cells displayed a characteristic chondrocyte morphology (Figure 1A). Immunohistochemistry showed that 72.9% of the implanted GFP⁺mPDC^{FGF2} expressed the master chondrogenic transcription factor SOX9 (Figure 1B). Moreover, numerous cells, especially at the periphery of the implant, had deposited collagen type II (Figure S2A). At this time point, blood vessels surrounded, but were not yet invading the implant (Figure 1C). Because the total diameter of the implants ranged from two to three millimeters, the maximal distance between the implanted cells and host vasculature was not more than 1.5 millimeter. Accordingly, cell survival was high, evidenced by the few TUNEL-positive implanted cells (1.2%; Figure 1D). On the other hand, cell proliferation was limited, as only 1.5% of GFP⁺mPDC^{FGF2} stained positive for the S/G2 marker phosphorylated histone H3 and just 3.3% of mPDC^{FGF2} had incorporated BrdU that was injected 6 hours prior to sacrifice (Figure S2B). In addition, imaging these constructs repeatedly during the first days after implantation using intravital microscopy showed that most GFP⁺mPDC^{FGF2} (92.5%) were detected each time at the same location, suggesting that cell migration was minimal, without specific direction, and occurring in random cells (Figure S2C). The speed of the migrating cells was low as well, which is consistent with the slow migration observed in other skeletal cells^(34,35).

To further investigate the dynamics of blood vessel ingrowth into the cartilage intermediates, we used intravital microscopy after fluorescent dextran injections. Vascular invasion of the

implant was not observed until one week after implantation (Figure 1E). This time point coincided with the appearance of mPDC^{FGF2} expressing collagen type I at the borders of the implant, evidenced by using mPDC^{FGF2} isolated from Col1a1-dsRed mice (Col1⁺). Histological analysis confirmed that invasion of blood vessels further into the cartilage intermediate was associated with progressively more Col1⁺ donor cells (Figure 1F). The close proximity of Col1⁺ donor cells to blood vessels (Figure 1G) suggests a tight association between angiogenesis and osteogenesis during endochondral bone regeneration in tissue-engineered constructs.

To assess the contribution of implanted versus host cells to bone formation, each cell source expressed a different fluorescent Col1a1 reporter. Until 14 days after implantation, the majority of the bone-forming cells was donor-derived (Figure 1H), whereas at day 21 this ratio had shifted strongly towards host-derived osteoblasts. These data indicate that bone formation is initiated by the implanted cells that become osteoblasts, followed by massive invasion of host-derived osteogenic cells.

The endochondral potential of mBMSC, another skeletal cell type involved in bone repair, was also investigated in this model. Compared to mPDC, these cells deposited less glycosaminoglycans in micromass cultures supplemented with TGF- β 1 (Figure S2D). Implantation of mBMSC^{FGF2} resulted in less cartilage and bone formation at day 12, compared to mPDC^{FGF2} implants, and more fibroblast-like cells were observed (Figure S2E). These findings are in line with our previous observations showing that mPDC^{FGF2} have higher endochondral potential ⁽²⁸⁾. Only in the mPDC^{FGF2} implants the endochondral ossification process continued until the cartilage template was entirely vascularized and converted into mature bone and bone marrow, a process that was completed between three and four weeks after implantation (Figure S2F) ⁽²⁸⁾. Taken together, these findings indicate that chondrogenically primed mPDC can survive in an avascular environment for several days and

that the arrival of blood vessels initiates bone formation, first by donor osteogenic cells and later by host cells.

3.2 Upscaling of cartilage intermediates impairs their regenerative outcome

The lack of blood vessels during the first days implies that the implanted cells rely on passive diffusion of nutrients and oxygen from the surrounding host vasculature. We therefore questioned whether chondrogenically primed cells can still survive and function in larger implants where mass transport to the central region becomes limited. To this end, cartilage intermediates with a threefold larger volume were generated. At day 12 after implantation, cartilage formation, chondrocyte hypertrophy and osteogenesis were detected at the periphery, but in the center only cells with fibroblast-like morphology were observed (Figure 2A). Staining for SOX9 revealed that most cells at the periphery expressed this chondrocytic marker, whereas SOX9-positive cells were not observed in the central regions of large implants (Figure 2B).

At this time point, blood vessels were growing into the implant and few blood vessels were also observed in the center of the implant that had become fibrous tissue (Figure 2C). These findings indicate that vascularization of a large implant was associated with endochondral ossification but only at the periphery and that coupling between angiogenesis and osteogenesis was lacking in the center. Consequently, μ CT analysis at day 21 revealed that mineralized tissue was not formed in the central regions, while small implants contained mineralized tissue throughout (Figure 2D). Taken together, these results indicate that mPDC^{FGF2} can potentially induce endochondral bone formation in an avascular environment, but they are unable to maintain a chondrocyte phenotype and promote endochondral ossification when they are located too far from the host vasculature for extended periods.

3.3 High VEGF levels do not alter differentiation of mPDC^{FGF2} *in vitro*

A frequent strategy to stimulate vascularization in tissue-engineered implants is to administer angiogenic growth factors, with VEGF being commonly used ⁽¹⁷⁾. However, since cartilage is avascular, it remains unclear whether stimulation of angiogenesis would promote or hinder endochondral bone tissue engineering. We therefore assessed the effects of VEGF overexpression in mPDC on their endochondral bone-forming capacity. As many skeletal mesenchymal cells express VEGF receptors and VEGF may alter their differentiation capacity ⁽²⁰⁾, we first analyzed *in vitro* whether VEGF influenced the chondrogenic and/or osteogenic potential of mPDC^{FGF2} directly. Compared to controls, adenovirus-mediated overexpression resulted in twofold higher secreted VEGF levels, starting early after transduction and lasting for at least 3 weeks (Figure 3A). In control conditions, VEGF levels also increased somewhat over time, which correlated with cultures becoming confluent (Figure 3A). DNA measurements revealed that exposure to these increased VEGF levels did not alter cell numbers (Figure 3B), suggesting that cell survival and/or proliferation were not altered.

We next performed chondrocyte differentiation assays. Alcian Blue staining of mPDC^{FGF2} cultured in micromasses with TGF- β 1 showed that similar amounts of glycosaminoglycans were deposited in control and VEGF-overexpressing cells (VEGF^{high} mPDC^{FGF2}; Figure 3C). In agreement, mRNA levels of the cartilage markers *Sox9*, collagen type II (*Col2*) and aggrecan (*Acan*) did not differ significantly between control and VEGF^{high} mPDC^{FGF2} (Figure 3D). Of note, the expression of *Col2* in VEGF^{high} mPDC^{FGF2} was already increased at the start of chondrogenic differentiation and did not change further during differentiation (data not shown). Culture in osteogenic conditions followed by staining for alkaline phosphatase or Alizarin Red showed that overexpression of VEGF did not affect the differentiation of mPDC^{FGF2} into osteoblasts (Figure 3E). These results were confirmed by gene expression analysis, showing that the osteoblast markers osterix (*Osx*) and osteocalcin (*Ocn*) were expressed similarly in control and VEGF^{high} mPDC^{FGF2} following osteogenic differentiation (Figure 3F). Thus, the

high VEGF levels do not interfere with *in vitro* chondrogenic or osteogenic differentiation of mPDC^{FGF2}.

3.4 VEGF enhances *in vivo* angiogenesis and osteogenesis at early time points

To determine whether the increased VEGF availability promotes endochondral bone formation *in vivo*, we started with implanting small volumes (100 μ l) of collagen-embedded control and VEGF^{high} mPDC^{FGF2}. At day 3, implants were recovered and H&E-staining showed that numerous cells in the two conditions displayed a chondrocyte morphology (Figure 4A). In agreement, 76.4% of the implanted control and 77.1% of VEGF^{high} mPDC^{FGF2} expressed SOX9 (Figure 4B). Furthermore, proliferation, as measured through phosphorylated histone H3-positive cells, was similar in both constructs (around 1.5%; Figure 4C). These data indicate that the formation of the cartilage intermediate appeared similar in the two conditions at this early time point.

We next analyzed implants at day 7 and observed abundant deposition of collagen type II matrix in both conditions (Figure S3A). This time point marks the onset of vascular ingrowth in the control condition. Indeed, CD31 staining showed that host blood vessels lined implants containing control mPDC^{FGF2}, and few of them just started growing into the cartilage intermediate (Figure 4D). As expected, the number of blood vessels surrounding and already invading the implants with VEGF^{high} mPDC^{FGF2} was manifestly higher at this time point (10.8 fold, $P<0.001$; Figure 4D). This increase in angiogenesis coincided with enhanced differentiation of donor cells into the osteogenic lineage as evidenced by the increased number of Col1⁺ cells (2.7 fold, $P<0.01$; Figure 4D), but was not associated with increased co-invasion of Col1⁺ host cells (Figure S3B). These findings suggest a beneficial effect of VEGF on the early phases of endochondral bone formation in chondrocytic implants.

3.5 VEGF overexpression leads to fibrosis at the center at late time points

To investigate the long-term effects of high VEGF levels on bone regeneration, implants containing control or VEGF^{high} mPDC^{FGF2} were analyzed at day 12. *Ex vivo* μ CT analysis showed that 26.6% more mineralized tissue was formed at this time point in implants with VEGF^{high} mPDC^{FGF2} ($P < 0.01$; Figure 5A). Histological analysis on H&E-stained sections confirmed the larger region of mature bone enclosing bone marrow that was formed at the periphery in VEGF^{high} implants (Figure 5B). However, a less favorable evolution was noticed in the center of the VEGF^{high} implants, as this region was devoid of cartilaginous tissue and contained mainly fibrous tissue (Figure 5B, Figure S3C-D). These observations were confirmed by reticulin staining showing that the center of VEGF^{high} implants was filled with reticular fibers, which were not detected in the bone marrow at the periphery of these constructs nor in the control implants (Figure 5B).

To determine whether the fibrotic tissue was formed by the implanted cells or by host cells, a similar experiment was performed with mTomato-labeled VEGF^{high} mPDC^{FGF2} implanted into GFP-expressing hosts. Histological analysis showed that mTomato-positive mPDC^{FGF2} were present in the remaining cartilage template at the periphery, but that almost the entire central fibrotic tissue was made up by host-derived cells (>98% of cells; Figure S4A). This effect was accompanied by invasion of blood vessels into the center of the VEGF^{high} implants, an effect not observed in control implants (Figure S4B). In addition, staining for collagen type II revealed that control implants still contained abundant cartilaginous matrix in the center, whereas almost no cartilage template remained in the VEGF^{high} constructs (Figure 5C, Figure S3D). Thus, enhanced invasion of blood vessels into the center of the implant results in replacement of the cartilaginous matrix by fibrotic tissue.

To investigate whether this fibrotic core delays or rather impedes bone formation at later time points, constructs were implanted for 21 days. In implants containing control mPDC^{FGF2} the

cartilage tissue was almost completely replaced by mature bone and bone marrow with only a few cartilage remnants persisting (Figure 5D). In contrast, implants with VEGF^{high} mPDC^{FGF2} contained mature bone and bone marrow at the periphery, but the center consisted of fibrotic tissue, where occasionally a few small bone spicules were observed. These results were confirmed by *ex vivo* μ CT, showing a comparable amount of total mineralized tissue volume between the conditions, but absence of mineralized matrix in the central regions of VEGF^{high} implants (Figure 5E). Similar data were obtained after 26 days of implantation. Because VEGF overexpression induced fibrosis already in small scaffolds, we did not investigate this strategy in larger implants. Taken together, these results show that a moderate but prolonged increase in VEGF enhances angiogenesis and osteogenesis at the periphery of chondrocytic implants, but causes enhanced blood vessel invasion leading to fibrosis in the center of these implants, thereby precluding endochondral bone formation at this site.

3.6 Enhanced hypoxia signaling preserves the endochondral cartilage pathway

An alternative strategy to enhance bone formation in an avascular environment is to modulate the hypoxia signaling pathway. This pathway induces protective responses that affect not only angiogenesis, but also cellular metabolism, redox homeostasis and survival ⁽³⁾. To investigate whether hypoxia signaling could enhance endochondral bone formation in tissue-engineered constructs, we knocked down its main negative regulator, PHD2, in mPDC^{FGF2} prior to implantation, resulting in HIF-1 α , but not HIF-2 α , stabilization (PHD2^{KD} mPDC^{FGF2}; Figure S5). These mPDC^{FGF2} were then embedded in large volumes of collagen (300 μ l) and implanted subcutaneously for 12 days, the time point at which vascular invasion is present. As expected, histological analysis showed that the center of control implants had become fibrotic (Figure 6A, Figure S6A). In contrast, centrally localized PHD2^{KD} mPDC^{FGF2} still displayed a chondrocyte-like morphology in large implants. This observation was confirmed by SOX9

immunohistochemistry, showing abundant SOX9-positive cells in the center of PHD2^{KD} implants, but only a few in control implants (35% and 12% respectively, $P<0.01$; Figure 6B). Furthermore, collagen type II-positive cartilage matrix was largely undetectable in the central fibrotic regions of control constructs, but was clearly present in the center of PHD2^{KD} implants (Figure 6C, Figure S6A). At the periphery, the abundance of collagen type II was comparable between the two conditions. These data indicate that chondrocyte characteristics were better preserved in the center when HIF signaling was induced.

As increased HIF signaling reduces oxygen consumption and thereby decreases hypoxic stress in periosteal cells ⁽³⁾, sections were stained for pimonidazole adducts. This analysis revealed that in implants at day 12 only few PHD2^{KD} mPDC^{FGF2} showed reductive metabolism, characteristic of hypoxic stress, whereas hypoxic cells were much more abundant in controls (0.7% and 6.9% respectively, $P<0.01$; Figure 6D). The increased hypoxic stress did not affect cell survival manifestly, as TUNEL staining showed that cell death was relatively low in both types of implants, although a trend towards higher survival of PHD2^{KD} mPDC^{FGF2} was noticed (Figure S6B). At later time points, however, the cell number per tissue area was higher in the non-vascularized regions of large PHD2^{KD} mPDC^{FGF2} implants compared to controls (50.5 versus 23.8 cells per field respectively, $P<0.001$; Figure S6C), suggesting improved cell survival. To determine whether angiogenesis contributed to the maintenance of the chondrogenic phenotype, CD31 staining was performed at day 12 after implantation. This analysis showed that the average blood vessel density did not differ significantly between control and PHD2^{KD} mPDC^{FGF2} implants (Figure S6D). These observations suggest that increased HIF signaling in chondrogenic cells protects the chondrogenic properties mainly via cell-autonomous mechanisms, and not through angiogenesis. To further substantiate this hypothesis, *in vitro* chondrogenic differentiation in micromasses was performed. No significant difference in gene expression was observed at the start (data not shown), but PHD2^{KD}

mPDC^{FGF2} had deposited more Alcian Blue-positive matrix after 9 days (26% increase in absorbance, $P<0.05$; Figure 6E) and significantly upregulated gene expression of the cartilage markers *Sox9*, *Col2* and *Acan* (1.5-, 2.5- and 3-fold respectively; Figure 6F) compared to control mPDC^{FGF2}. Together, these results suggest that activation of hypoxia signaling prior to implantation enhances the chondrogenic capacity of mPDC^{FGF2} and increases their ability to form and maintain endochondral cartilage in an avascular environment.

3.7 Activation of hypoxia signaling enhances bone formation in large implants

To determine whether the increase in cartilage abundance in PHD2^{KD} implants also leads to more bone formation, large implants were examined at day 21 after implantation. Control implants contained mature bone with bone marrow at the periphery, but the center consisted mainly of fibrous tissue (Figure 7A). In contrast, large parts of the PHD2^{KD} implants consisted of mature bone and bone marrow, and the soft tissue in the center was mainly cartilaginous. The increase in mature bone mass was associated with vascularization of a larger area in PHD2^{KD} implants, as evidenced by CD31 staining, whereas blood vessels were scarce in the central fibrous region of control implants (116.5 vs. 65.6 respectively, $P<0.01$; Figure 7B). Accordingly, *ex vivo* μ CT showed that control constructs had a mineralized outer shell but contained almost no mineralized tissue in the center, whereas PHD2^{KD} implants consisted of significantly more mineralized tissue distributed throughout the entire construct (Figure 7C). Together, these data show that the enhanced chondrogenic differentiation of PHD2^{KD} mPDC^{FGF2} leads to improved endochondral bone formation at later time points, especially in the central regions of the implant that otherwise become fibrotic. Next, the beneficial effect of enhanced hypoxia signaling on bone formation was also confirmed in an orthotopic large bone defect. PHD2^{KD} mPDC^{FGF2} were seeded onto calcium/phosphate-collagen scaffolds and implanted in a non-healing tibial bone defect for 8 weeks. Analysis by μ CT showed that

significantly more mineralized tissue was formed in PHD2^{KD} versus control implants at 8 weeks after implantation (46.7% vs. 35.6% respectively, $P<0.05$; Figure 7D). Finally, to investigate the translational potential of this strategy, mPDC^{FGF2} were treated for three days prior to implantation with the PHD inhibitor IOX2 to activate the hypoxia signaling pathway (Figure S7). Confirming the genetic approach, pharmacological blockade of PHDs maintained cartilage integrity in the construct center and stimulated bone formation without the formation of fibrous tissue (Figure 8A-C). These findings further confirm the potential of hypoxia signaling modulation as a strategy to enhance tissue-engineered bone regeneration.

4. DISCUSSION

A major drawback in regenerative medicine is that the size of the engineered implant that can be used is still limited and this problem is largely caused by the poor vascularization at the implant site ⁽³⁶⁾. In bone regeneration, tissue-engineered cartilage intermediates represent an interesting solution for this issue, because chondrocytes can survive and function in an avascular environment ⁽¹⁴⁾. However, we now demonstrate that increasing the size of cartilage intermediates ($> 100 \text{ mm}^3$) results in loss of chondrogenic characteristics and replacement of the cartilage by fibrotic tissue in the center of the implant. As a therapy, supplementation of VEGF is not advisable, because although it increases vascularization it also interferes with the endochondral process of replacing cartilage by bone. In contrast, activation of hypoxia signaling prior to implantation enhances chondrogenesis without causing fibrosis and thus results in improved bone formation in large tissue-engineered constructs.

Hypoxia occurs in the enlarging avascular growth plate during bone development ⁽²³⁾, and analogously, increasing the size of tissue-engineered cartilage intermediates leads to hypoxic cells in the center of the implant. This stress condition did not cause manifest cell death in the implant, an effect that, on the other hand, has been observed in models of direct bone formation ^(1,2,3). However, the formation of a dense collagen type II matrix, an energy-demanding anabolic process, was compromised. Consequently, fibrotic tissue was formed, resulting in a poorly mineralized center.

Stimulation of angiogenesis did not improve endochondral bone formation in the cartilage intermediates, but rather disturbed this coordinated process and also resulted in fibrosis. High levels of VEGF are known to contribute to fibrosis in the lung ⁽³⁷⁾, retina ⁽³⁸⁾, and even hematopoietic bone marrow ⁽³⁹⁾. We now show that it can have deleterious effects during

endochondral ossification as well. Factors that may have contributed to this adverse outcome include premature degradation of the cartilage matrix and lack of osteoprogenitors when blood vessels invade the center. More precisely, the amount of collagen type II matrix that was deposited in the two conditions at an early time point (day 7) was comparable. However, a few days later, the center of the implants with high VEGF expression was devoid of collagen type II matrix, which coincided with blood vessel invasion into the central region. These findings suggest that the increased abundance of blood vessels induces more degradation of the, possibly less mature, collagen type II matrix. The invading endothelial cells and chondroclasts are indeed considered to contribute to the degradation of the cartilage matrix, as blocking vascular invasion of the hypertrophic chondrocyte zone during development results in an enlarged growth plate ⁽⁴⁰⁾. In addition, this enhanced vascular invasion of the center may be insufficiently accompanied by co-migrating osteoprogenitors: more donor mPDC are differentiating to osteoblasts at the borders of the implant at early time points and these differentiated cells do not migrate, whereas the denser vascular network was not associated with increased invasion of host osteoprogenitors. Together, these processes induce an uncoupling of cartilage degradation and initiation of osteogenesis. These results are in agreement with the observations that inhibition of angiogenesis can promote stable cartilage formation ^(20,41). They also support the notion that vascularization can be beneficial for endochondral bone repair ⁽¹⁵⁾, but only when timing is adequate.

The formation of a fibrotic core was observed in two conditions: (i) when the size of the implant was increased and (ii) when VEGF-overexpressing cells were implanted. A remarkable similarity between these two fibrotic outcomes is the preceding lack of cartilage matrix, indicating that sufficient matrix is crucial to sustain endochondral ossification ^(42,43). These data also suggest that using a cartilaginous matrix as a scaffold may be beneficial to drive

endochondral bone repair. Decellularized matrix has clinical potential in regenerative therapies for heart, tendons, and even articular cartilage ⁽⁴⁴⁾, but has remained relatively unexplored in endochondral bone tissue engineering ⁽¹⁰⁾. One study showed that decellularized hypertrophic cartilage template leads to endochondral ossification upon implantation, but not to the same extent as in implants still containing chondrocytes ⁽⁴⁵⁾. Likely, the combination of an appropriate matrix together with cells that are adapted to the unfavorable environment is required to improve the regenerative outcome. Of note, we confirm here that mPDC inherently possess more capacity for endochondral bone regeneration than mBMSC ⁽²⁸⁾, suggesting that periosteal cells may be better suited for tissue engineering applications.

In contrast to the negative effects of enhanced VEGF signaling, activation of hypoxia signaling prior to implantation promoted endochondral ossification in large cartilage intermediates. This beneficial effect was not caused by increased angiogenesis during the early stages, but by the preservation of chondrogenic properties and enhanced collagen type II matrix formation in the center. HIF-1 α is known to transcriptionally regulate SOX9 and enhance chondrogenesis during bone development ⁽⁴⁶⁾. Our data agree with this notion, as *Sox9* and other chondrogenic marker genes were upregulated in PHD2^{KD} cells *in vitro* and more SOX9-positive cells were observed *in vivo*.

In addition, the increased HIF signaling may adapt the implanted cells metabolically to help cells conserve energy and redox homeostasis in an avascular environment ⁽²²⁾. Indeed, activation of HIF signaling in osteogenic cells prior to implantation has been shown to improve direct bone formation by adapting cell metabolism. More precisely, more intracellular glycogen was formed, which was used as energy source, as well as more glutathione was synthesized, which preserved redox homeostasis ⁽³⁾. The specific metabolic needs of endochondral chondrocytes are however poorly characterized, but activation of HIF signaling likely helps these cells to

metabolically adapt to diffusion-limited levels of nutrients in the cartilage intermediates. In addition, a denser blood vessel network was observed at later stages when HIF signaling was activated. However, this effect was smaller than observed with VEGF overexpression and therefore did not disturb the coordinated process of cartilage replacement by bone. In line with previous observations, activation of hypoxia signaling in mPDC prior to implantation also enhanced bone regeneration in an eutopic large bone defect and pharmacologic targeting of the hypoxia signaling pathway was as effective as the genetic approach ⁽³⁾. Taken together, our data demonstrate that the activation of hypoxia signaling prior to implantation can enhance endochondral bone repair in large defects, indicating that this strategy may be beneficial in regenerative therapies.

5. CONCLUSION

In the current study, we demonstrate that, although chondrocytes can survive and function in an avascular environment, increasing the size of cartilage intermediates for treating large skeletal defects results in a fibrotic central core and impaired bone formation. Stimulation of angiogenesis enhances the conversion of cartilage into bone at the borders of the implant, but disturbs this process in the center resulting in a central fibrotic core. Hence, supplementation of VEGF is not advised to improve bone formation via tissue-engineered cartilage intermediates unless timed and dosed very carefully. In contrast, activation of hypoxia signaling before implantation increases cartilage formation and subsequent ossification in large implants. This approach could therefore be used to potentiate endochondral bone repair in large avascular defects and further improve the therapeutic potential of bone tissue engineering approaches.

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AUTHOR CONTRIBUTIONS

Study design: PJS, NvG and GC. Study conduct: PJS, SS, RVL and ST. Data analysis and interpretation: PJS, SS and GC. Drafting manuscript: PJS. Revising manuscript: SS, NvG and GC. Final approval of manuscript: PJS, SS, NvG, RVL, ST and GC.

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FIGURE LEGENDS

Figure 1. Early processes in cartilage intermediates formed by mPDC^{FGF2}. (A) H&E-staining of mPDC^{FGF2} cartilage intermediate at day 3. (B) SOX9 immunohistochemistry and quantification of SOX9-positive cells in GFP⁺ mPDC^{FGF2} implants at day 3 (n = 6). (C) Immunohistochemistry for CD31 on mPDC^{FGF2} implants at day 3. (D) TUNEL staining and quantification of positive cells in GFP⁺ mPDC^{FGF2} implants at day 3 (n = 6). (E) Intravital imaging of vasculature, after perfusion with FITC-labeled dextrans, and of Colla1-dsRed⁺ mPDC^{FGF2} (Coll⁺) as marker of osteoblast differentiation (representative images of 4 mice are shown). At day 3, no blood vessels or osteoblasts were observed (top panel), but blood vessels started to grow into the tissue at day 7, closely associated with the appearance of Coll⁺ cells (arrowheads; bottom panel). Asterisks mark the implant. (F) Histological analysis of implants with Coll⁺ cells (from panel E) at day 7. CD31 immunohistochemistry and H&E-staining were performed sequentially on the same section to visualize Coll⁺ cells, endothelial cells, and tissue morphology. Right panel: higher magnification. (G) Quantification of the distance between Coll⁺ cells and the closest CD31⁺ blood vessel (n = 172 cells counted in 6 implants). (H) Images and quantification of Coll1-expressing donor cells, after transduction with Colla1-GFP, versus host cells, expressing Colla1-dsRed, at days 7, 14 and 21 after implantation (n = 4 per time point). Graph shows relative contribution of these two cell populations to bone formation at indicated time points. White dotted lines indicate the border of the implant, ca: cartilage. Numbered boxed areas were enlarged. Scale bars are 100 μ m (A-D, F-G) or 20 μ m (B, D: higher magnifications; E).

Figure 2. Larger volume of cartilage intermediates impairs bone formation. (A) H&E-staining on mPDC^{FGF2} cartilage intermediates recovered at day 12. (B) SOX9 immunohistochemistry and quantification of SOX9-positive cells in the periphery (peri; <1 mm from border) and center of large implants at day 12 (n = 4). (C) H&E-staining and CD31 immunohistochemistry on large implants at day 12. (D) μ CT visualization and quantification of mineralized tissue volume over total tissue volume (MV/TV) in small and large implants recovered at day 21 (n = 4). The center region is a circle with 1 mm diameter in the center of the implant. White dotted lines indicate the border of the implant, ca:

cartilage, f: fibrous tissue. Numbered boxed areas were enlarged. * = $P < 0.05$. Scale bars are 100 μm (A-C), 20 μm (A, C: additional insets) or 1 mm (D).

Figure 3. Characterization of VEGF-overexpressing mPDC^{FGF2} *in vitro*. (A) VEGF levels in medium of cultured control (CTRL) and VEGF-overexpressing (VEGF^{high}) mPDC^{FGF2} over 21 days, measured by ELISA (n = 3). (B) Cell growth, measured by DNA quantification of control and VEGF^{high} mPDC^{FGF2} seeded at equal cell density and cultured for 6 days (n = 3). (C) Alcian Blue staining of micromass cultures of control and VEGF^{high} mPDC^{FGF2}. Staining intensity was quantified in arbitrary units (AU) after dye extraction (n = 3). (D) Chondrogenic marker gene expression in micromasses of control and VEGF^{high} mPDC^{FGF2}, quantified by qRT-PCR (n = 3). (E) Alkaline phosphatase (ALP) and Alizarin Red (ALR) staining of control and VEGF^{high} mPDC^{FGF2} cultured in osteogenic medium. (F) Osteoblast marker gene expression following osteogenic culture of control and VEGF^{high} mPDC^{FGF2}, quantified by qRT-PCR (n = 3). * = $P < 0.05$ vs. control.

Figure 4. Early time points in VEGF-overexpressing cartilage intermediates. (A) H&E-staining of cartilage intermediates of control (CTRL) and VEGF^{high} mPDC^{FGF2} recovered at day 3. (B) SOX9 immunohistochemistry and quantification of SOX9-positive cells in control and VEGF^{high} implants at day 3 (n = 4). (C) Immunohistochemistry for phosphorylated histone H3 (H3-p) and quantification of H3-p-positive cells in control and VEGF^{high} implants at day 3 (n = 4). (D) Immunohistochemistry for CD31, visualization of Col1⁺ cells and H&E-staining on sequential sections of control or VEGF^{high} implants at day 7 (n = 6). Numbers of blood vessels and Col1⁺ cells were quantified. Insets show higher magnifications. White dotted lines indicate the border of the implant, ca: cartilage, b: bone. Numbered boxed areas were enlarged. * = $P < 0.05$ vs. control. Scale bars are 100 μm .

Figure 5. Bone formation is impaired in VEGF^{high} cartilage intermediates. (A) *Ex vivo* μCT analysis and quantification of mineralized volume over total tissue volume (MV/TV) in control (CTRL) and VEGF^{high} implants at day 12 (n = 6). The ratio of VEGF^{high} versus its control implant is also shown. (B) Reticulin staining of control and VEGF^{high} implants at day 12. (C) Immunohistochemistry for collagen

type II (COL2) of control and VEGF^{high} implants at day 12. **(D)** H&E-staining of control and VEGF^{high} implants at day 21. **(E)** *Ex vivo* μ CT analysis and quantification of mineralized volume over total tissue volume (MV/TV) in control and VEGF^{high} implants at day 21 (n = 4). The center region is a circle with 1 mm diameter in the center of the implant. Ca: cartilage, b: bone, f: fibrous tissue. Numbered boxed areas were enlarged. * = $P < 0.05$ vs. control. Scale bars are 100 μ m (B-D), 20 μ m (B: additional insets) or 1 mm (A, E).

Figure 6. Preservation of chondrogenic properties in large PHD2^{KD} implants at day 12. **(A)** H&E-staining of large implants with control (CTRL) or mPDC^{FGF2} with knock-down of PHD2 (PHD2^{KD}). **(B)** SOX9 immunohistochemistry of control and PHD2^{KD} implants. Quantification was performed in the center region starting 1 mm from the implant border (n = 6). **(C)** Immunohistochemistry for collagen type II (COL2) in large control and PHD2^{KD} implants. **(D)** Staining for and quantification of pimonidazole-positive cells in large control and PHD2^{KD} implants at day 12 (n = 4). **(E)** Alcian Blue staining and quantification of micromass cultures of control and PHD2^{KD} mPDC^{FGF2} (n = 3). **(F)** Gene expression of *Phd2* and chondrogenic markers in micromass cultures of control and PHD2^{KD} mPDC^{FGF2}, quantified by qRT-PCR (n = 3). Ca: cartilage, b: bone, f: fibrous tissue. Numbered boxed areas were enlarged. * = $P < 0.05$ vs. control. Scale bars are 100 μ m.

Figure 7. Improved bone formation in large PHD2^{KD} implants at day 21. **(A)** H&E-staining of large control (CTRL) or PHD2^{KD} implants. **(B)** Immunohistochemistry for CD31 and quantification of blood vessels in large control and PHD2^{KD} implants (n = 6). **(C)** *Ex vivo* μ CT analysis and quantification of mineralized volume over total tissue volume (MV/TV) in large control and PHD2^{KD} implants at day 21 (n = 5). **(D)** *Ex vivo* μ CT analysis and quantification of MV/TV in NuOss scaffolds seeded with control or PHD2^{KD} mPDC^{FGF2} and implanted in a critical tibial defect for 8 weeks (n = 4). Ca: cartilage, f: fibrous tissue. Numbered boxed areas were enlarged. * = $P < 0.05$ vs. control. Scale bars are 100 μ m (A-B) or 1 mm (C-D).

Figure 8. Pharmacological PHD inhibition preserves cartilage matrix and enhances bone formation in large implants. (A) Immunohistochemistry for collagen type II (COL2) of large implants with control (CTRL) or IOX2-treated mPDC^{FGF2} (IOX2) at day 7. (B) H&E-staining of large control or IOX2 implants at day 21. (C) *Ex vivo* μ CT analysis and quantification of mineralized volume over total tissue volume (MV/TV) in large control and IOX2 implants at day 21 (n = 6). B: bone, ca: cartilage, f: fibrous tissue. Numbered boxed areas were enlarged. * = $P < 0.05$ vs. control. Scale bars are 100 μ m (A-B) or 1 mm (C).

Supplementary figure 1. Negative controls for immunocytochemistry. For SOX9, CD31, TUNEL, type II collagen (COL2), pimonidazole adducts, phosphorylated histone H3 (H3-p) and BrdU immunostainings, the results with primary antibody (Ab.; top panels) and the negative control (CTRL; bottom panels) without primary antibody are shown. Hoechst staining for nuclear DNA is shown in the blue channel, the immunostainings are shown in the green (COL2) or red channel. For TUNEL staining the boxed area was magnified (top) and the individual color channels are shown (middle and bottom).

Supplementary figure 2, related to Figure 1. Characteristics of cartilage intermediates formed by mPDC^{FGF2}. (A) Immunohistochemistry for collagen type II (COL2) on implanted mPDC^{FGF2} at day 3. (B) Immunohistochemistry for phosphorylated histone H3 (H3-p) and bromodeoxyuridine (BrdU) incorporation, injected 6 hours prior to sacrifice. Graphs show quantification of H3-p-positive or BrdU-positive cells in implanted mPDC^{FGF2} at day 3 (n = 6). (C) Quantification of cell migration, assessed by intravital imaging of GFP⁺ mPDC^{FGF2} during the first 5 days after implantation (n = 3). Images were taken every 8 hours and aligned to the extracellular matrix visualized by second-harmonics generation (SHG). (D) Alcian Blue staining and quantification of micromass cultures of mPDC^{FGF2} and mBMSC^{FGF2} (n = 6). (E) H&E-staining on implanted mPDC^{FGF2} and mBMSC^{FGF2} at day 12 (F) H&E-staining and μ CT visualization of mineralized tissue in mPDC^{FGF2} implants at day 21. White dotted lines indicate the border of the implant, ca: cartilage, b: bone. Numbered boxed areas were enlarged. Scale bars are 100 μ m in all panels except E (20 μ m: additional insets) or F (1 mm: μ CT).

Supplementary figure 3, related to Figure 4 and Figure 5. mPDC^{FGF2} form osteoblasts in VEGF-overexpressing implants. (A) Immunohistochemistry for collagen type II (COL2) on control (CTRL) and VEGF^{high} mPDC^{FGF2} implants recovered at day 7. **(B)** Imaging of Col1-expressing donor versus host cells at the site of bone formation in VEGF^{high} implants at day 7. Top panel: VEGF^{high} donor cells express Col1a1-dsRed; bottom panel: host cells express Col1a1-dsRed. **(C)** H&E-staining of control and VEGF^{high} implants recovered at day 12. Insets show higher magnification. **(D)** Toluidine Blue staining of control and VEGF^{high} implants recovered at day 12. Insets show higher magnification. White dotted lines indicate the edge of the implant, ca: cartilage, b: bone, f: fibrous tissue. Numbered boxed areas were enlarged. Scale bars are 100 μ m.

Supplementary figure 4, related to Figure 4 and Figure 5. Host-dependent fibrosis in VEGF-overexpressing implants. (A) Histological analysis of mTomato-expressing VEGF^{high} mPDC^{FGF2} implanted into GFP-expressing hosts. Implants were recovered at day 12, and tissue morphology (H&E) and fluorescence (Hoechst) were visualized on adjacent sections. **(B)** Immunohistochemistry for CD31 on control (CTRL) and VEGF^{high} implants at day 12. Ca: cartilage, f: fibrous tissue. Numbered boxed areas were enlarged. Scale bars are 100 μ m (A-B) or 20 μ m (A: additional insets).

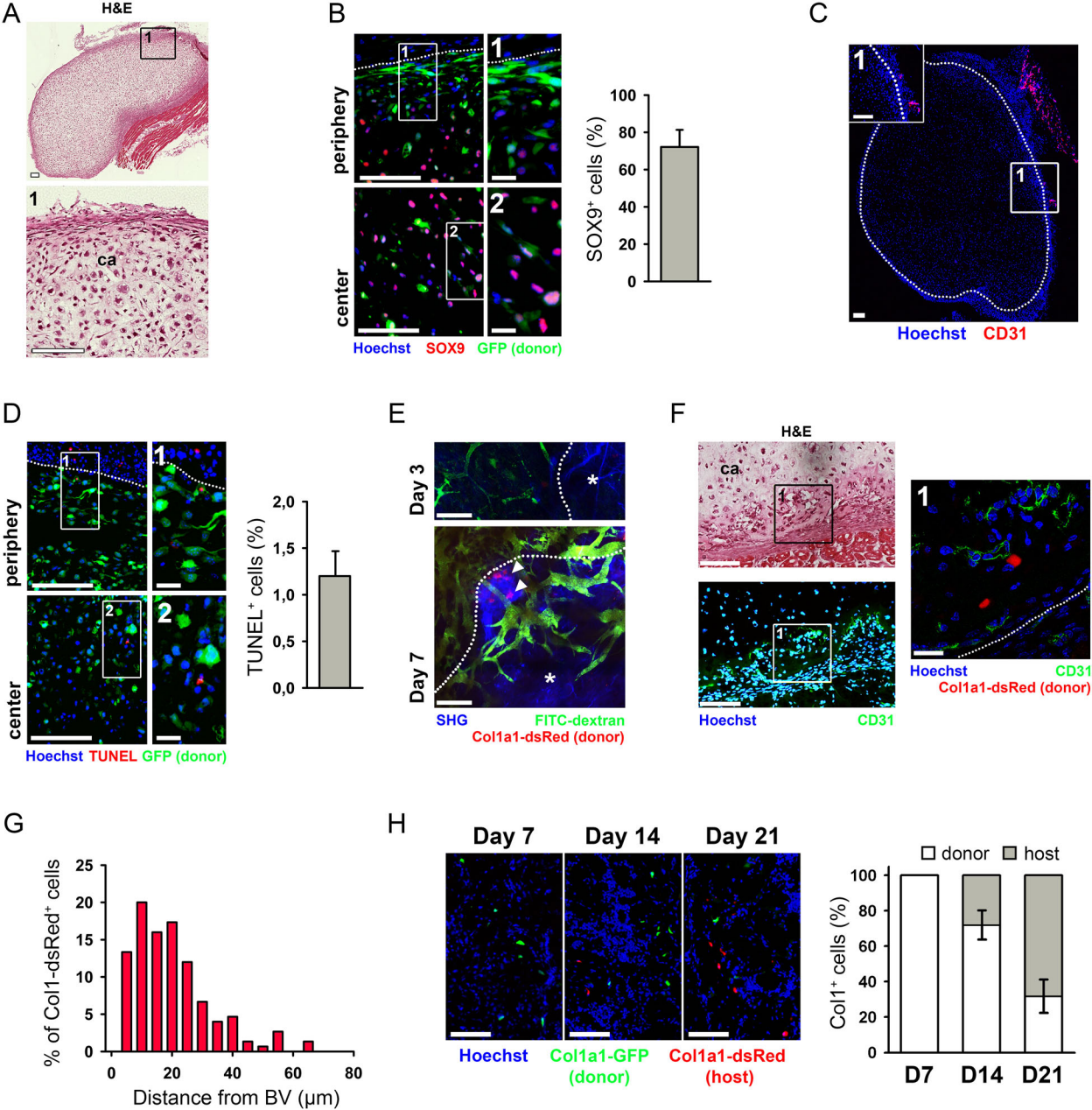
Supplementary figure 5, related to Figure 6. Genetic inactivation of PHD2 results in normoxic HIF-1 α stabilization. Gene expression and protein levels of PHD2 in control (CTRL) and PHD2^{KD} mPDC and its effect on HIF-1 α and HIF-2 α levels via Western blot analysis (representative images of n = 3 are shown). β -actin and Lamin A/C are loading controls. * = $P < 0.05$ vs. control.

Supplementary figure 6, related to Figure 6. Genetic activation of hypoxia signaling protects implanted mPDC^{FGF2} against hypoxic stress. (A) Toluidine Blue-staining of large implants with control (CTRL) or IOX2-treated mPDC (IOX2) at day 12. **(B)** TUNEL staining and quantification of positive cells in control and PHD2^{KD} implants at day 12 (n = 4). **(C)** Quantification of cell density in the center of large control and PHD2^{KD} implants at day 12 (left panels, n = 6) and day 21 (right panels, n = 6). **(D)** Immunohistochemistry for CD31 and quantification of blood vessels in large control and

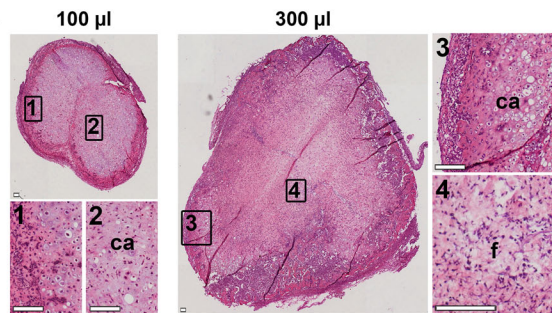
PHD2^{KD} implants at day 12 (n = 6). Ca: cartilage, f: fibrous tissue. Numbered boxed areas were enlarged.

* = $P < 0.05$ vs. control. Scale bars are 100 μm .

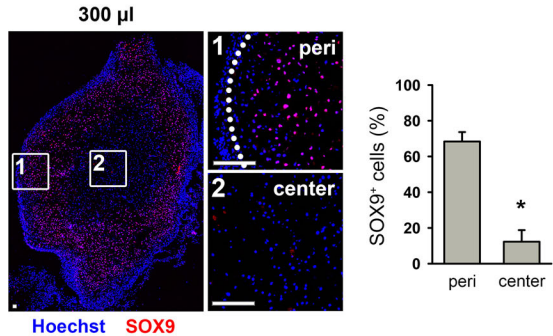
Supplementary figure 7, related to Figure 8. IOX2 treatment in mPDC^{FGF2} results in normoxic HIF-1 α stabilization. Protein levels of HIF-1 α and HIF-2 α in vehicle-treated control (CTRL) and IOX2-treated (IOX2) mPDC^{FGF2} via Western blot analysis (representative images of n = 3 are shown). Lamin A/C is loading control.



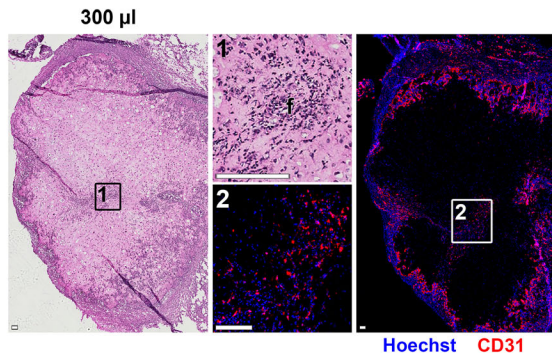
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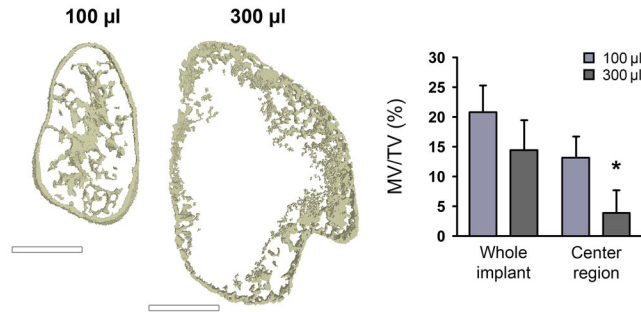
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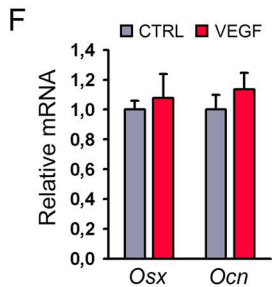
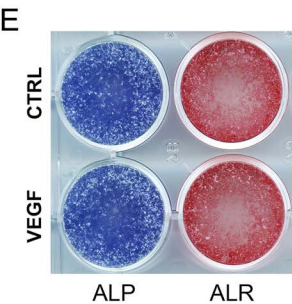
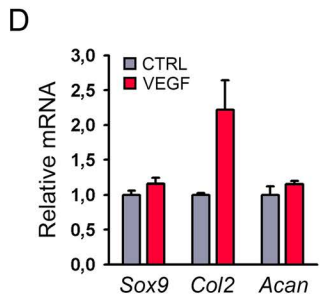
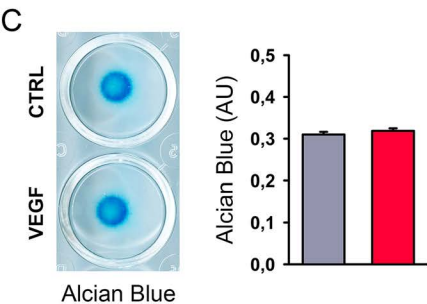
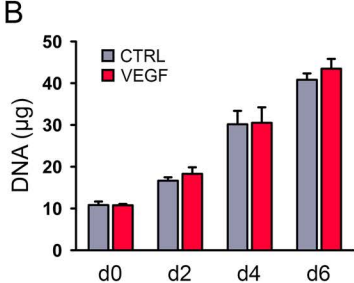
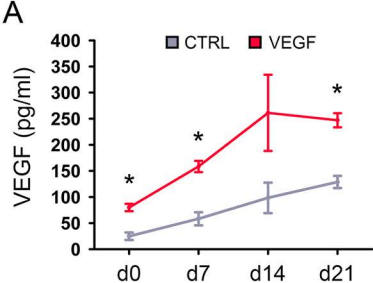


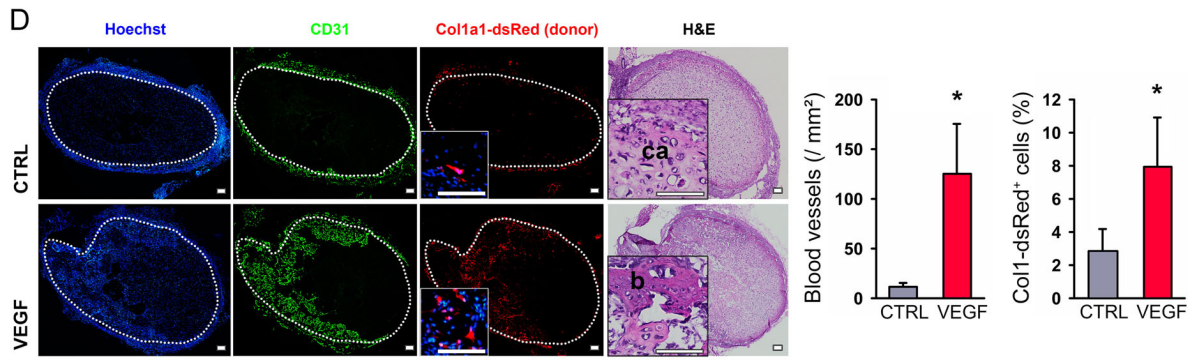
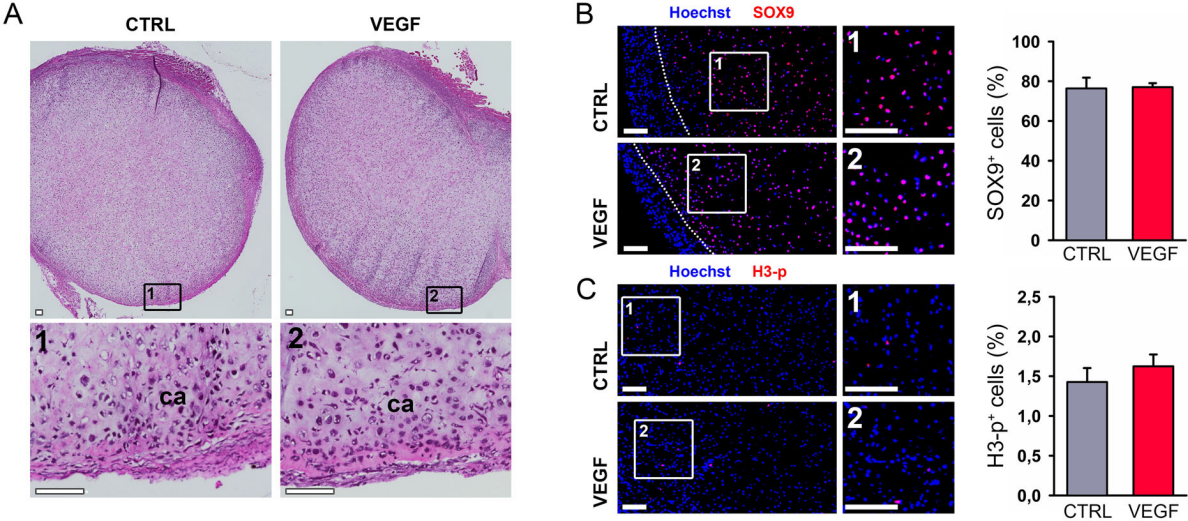
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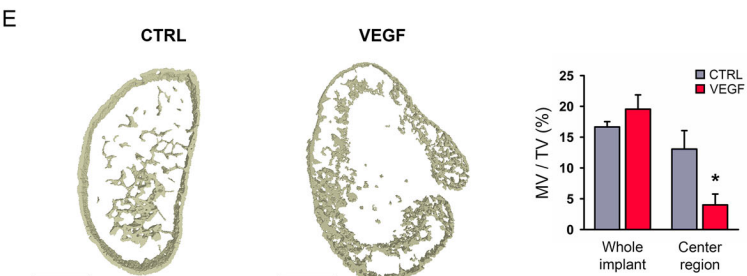
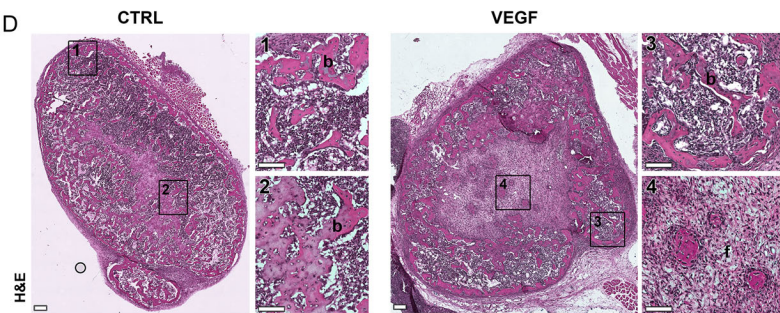
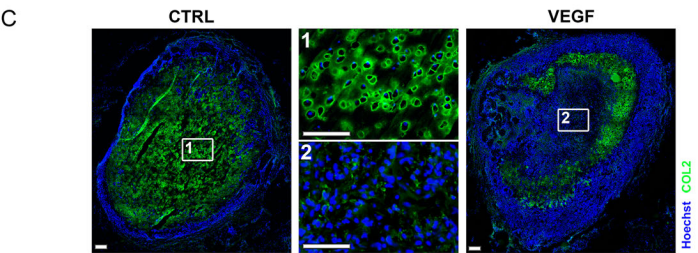
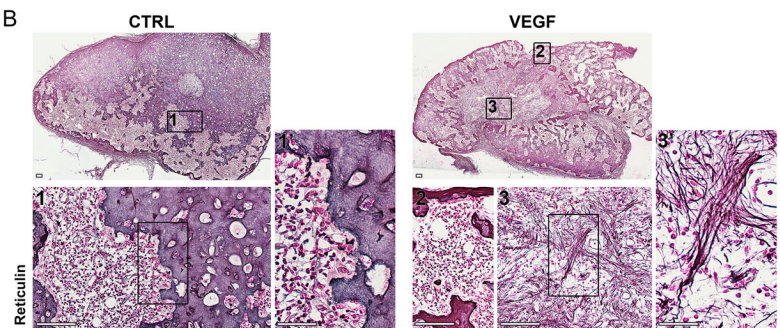
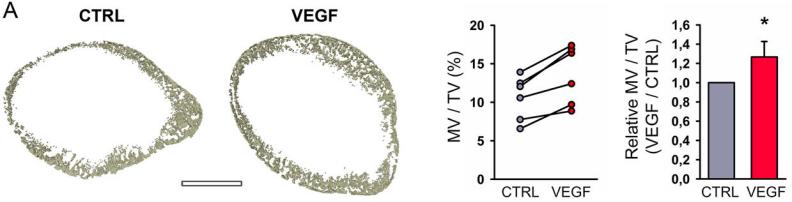


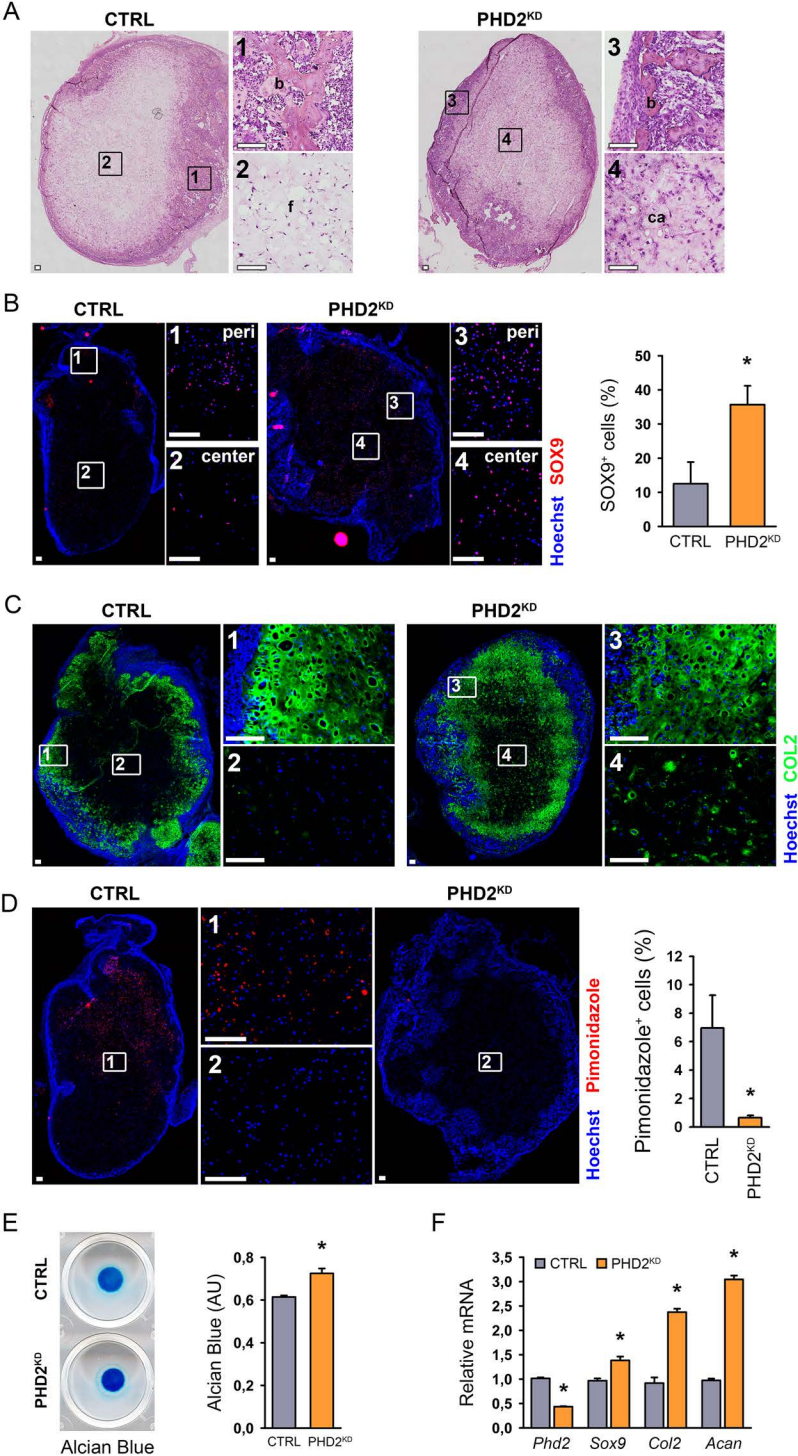
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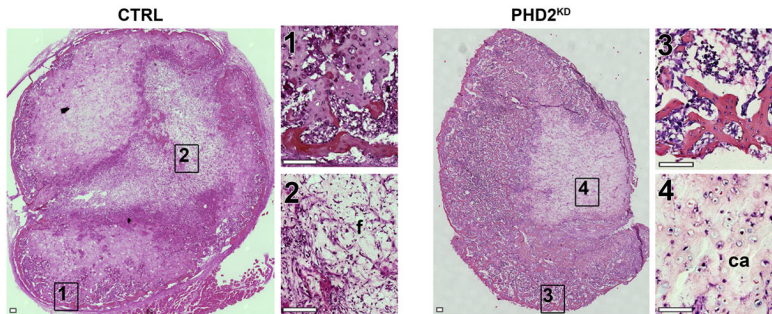




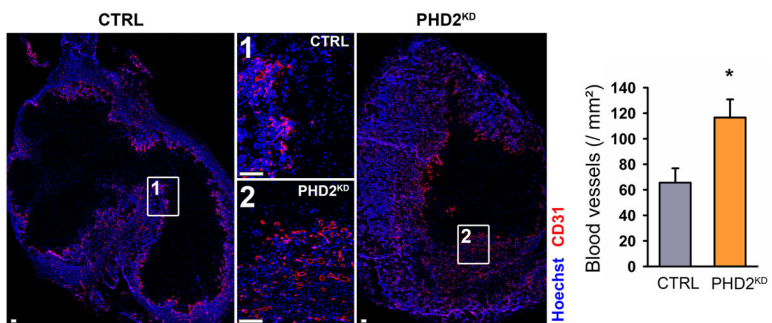




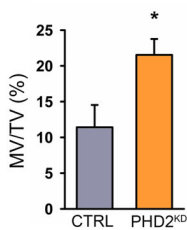
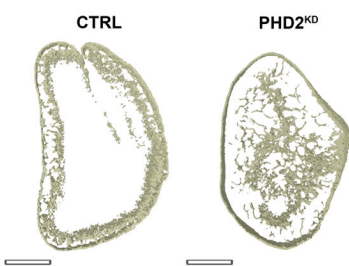
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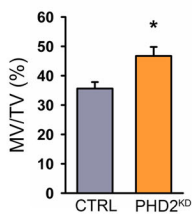
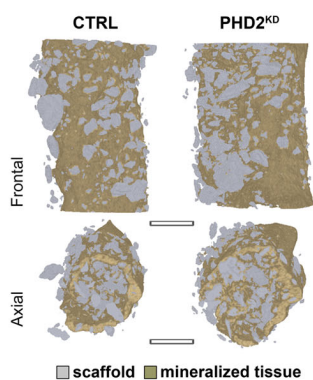
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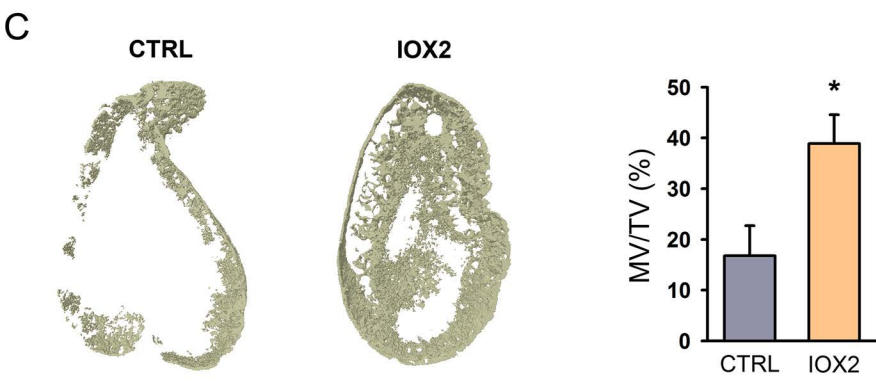
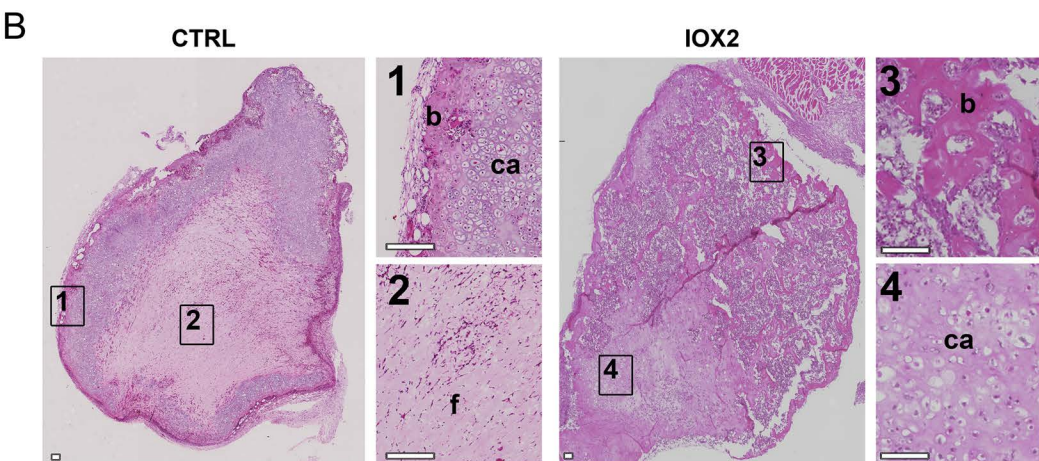
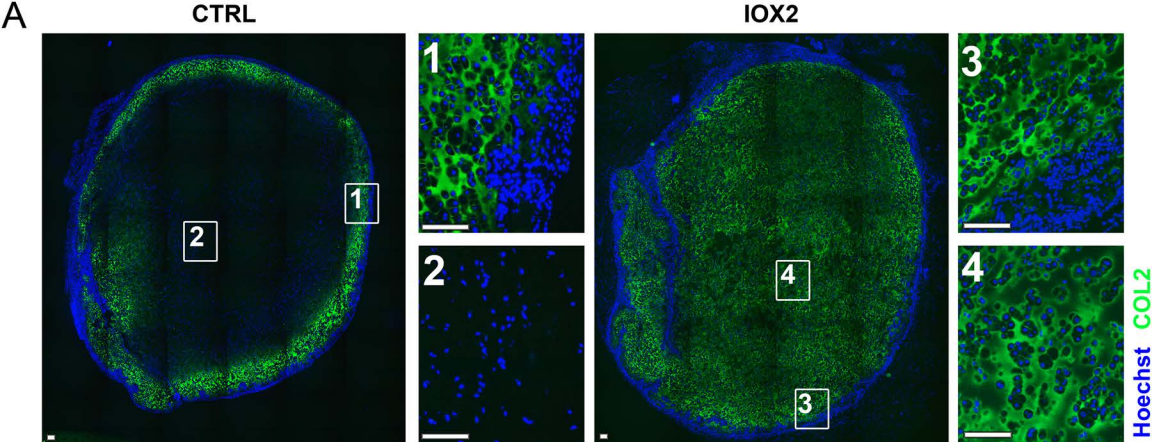


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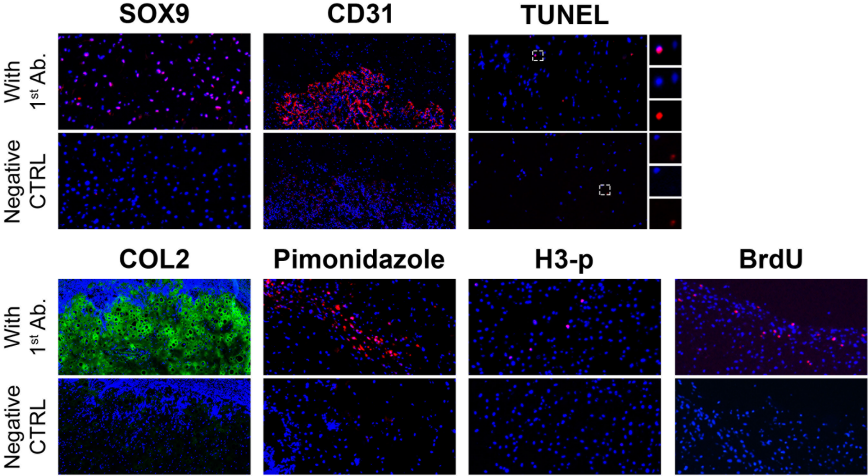
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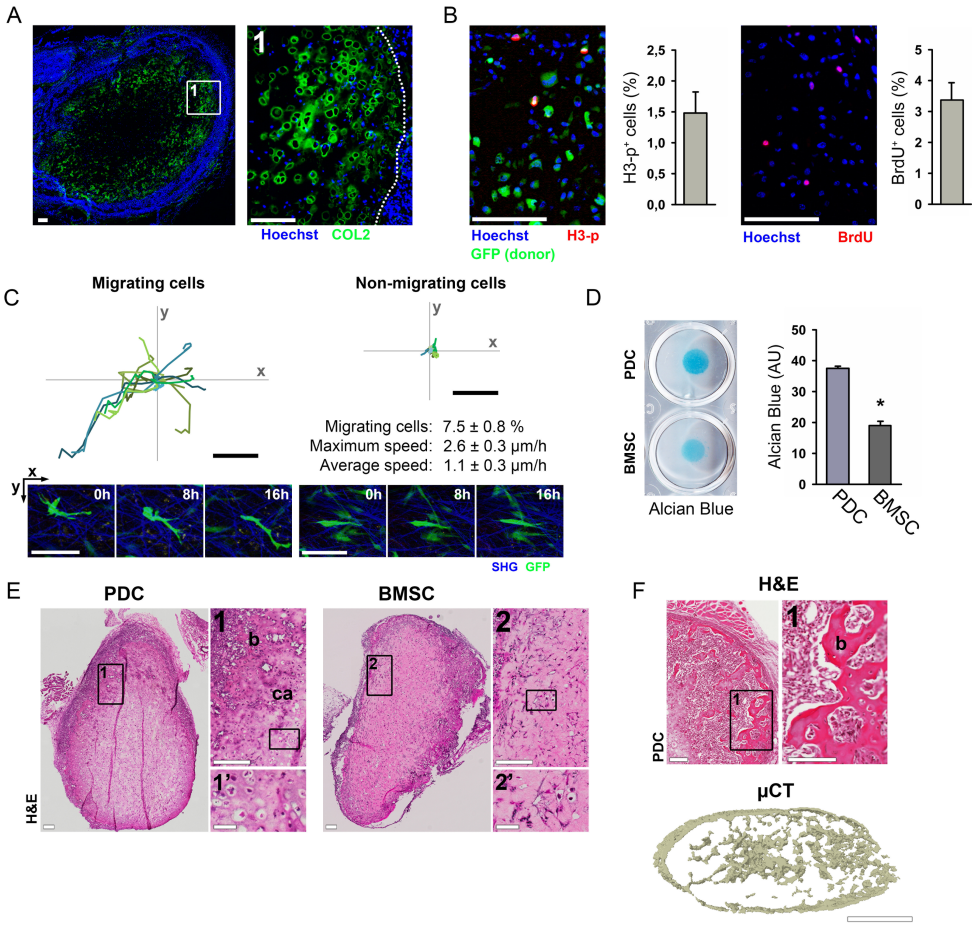


Cells	Description
mPDC	Murine periosteum-derived cells, isolated and cultured as described in van Gastel <i>et al.</i> (Stem Cells 2012)
mPDC ^{FGF2}	mPDC pre-treated with fibroblast growth factor 2, cultured as described in van Gastel <i>et al.</i> (Stem Cells 2014)
mBMSC	Murine bone-marrow derived cells, isolated and cultured as described in van Gastel <i>et al.</i> (Stem Cells 2014)
mBMSC ^{FGF2}	mBMSC pre-treated with fibroblast growth factor 2, cultured as described in van Gastel <i>et al.</i> (Stem Cells 2014)
VEGF ^{high}	mPDC ^{FGF2} transduced with adenovirus expressing vascular endothelial growth factor A, 164 amino acid isoform
PHD2 ^{KD}	mPDC ^{FGF2} from <i>Phd2</i> ^{fl/fl} mice treated with adenovirus expressing Cre resulting in knockdown of prolyl hydroxylase domain-containing protein 2, as described in Stegen <i>et al.</i> (Cell Metabolism 2016)
GFP ⁺	mPDC constitutively expressing (enhanced) green fluorescent protein
Col1 ⁺	mPDC actively expressing the Col1a1-dsRed or Col1a1-GFP osteogenic reporter

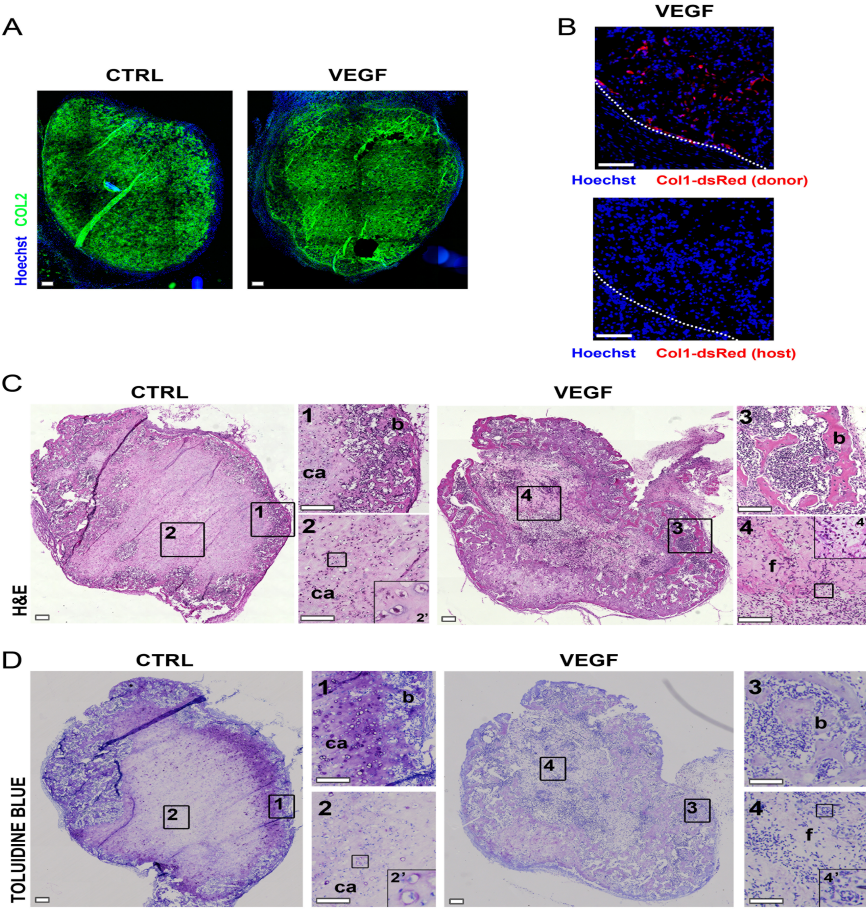
Supplementary Figure 1



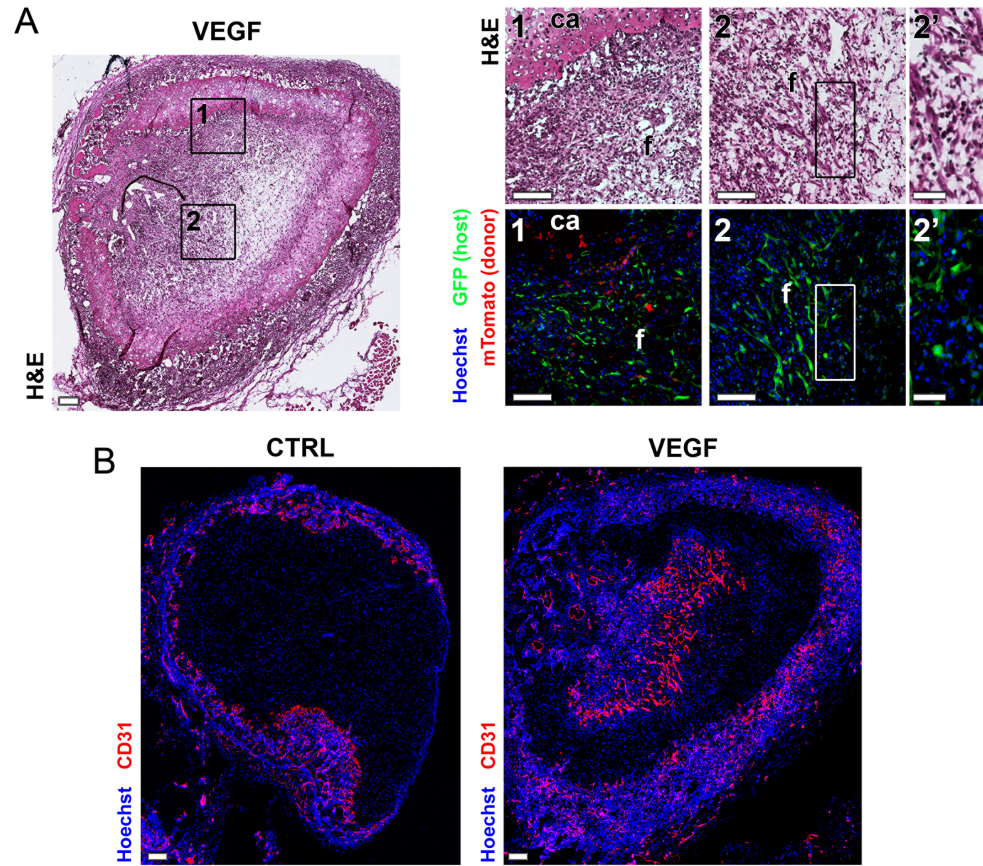
Supplementary figure 2



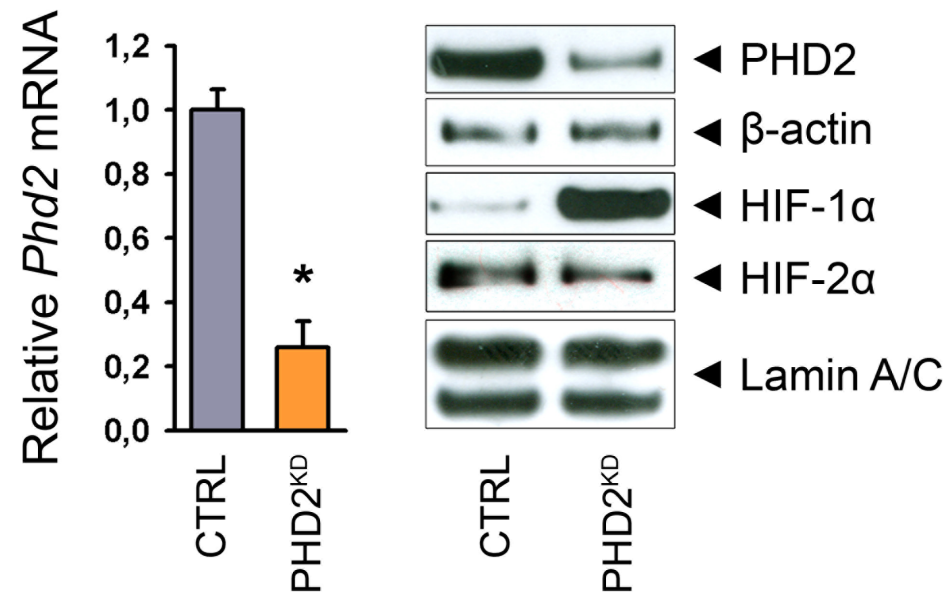
Supplementary figure 3



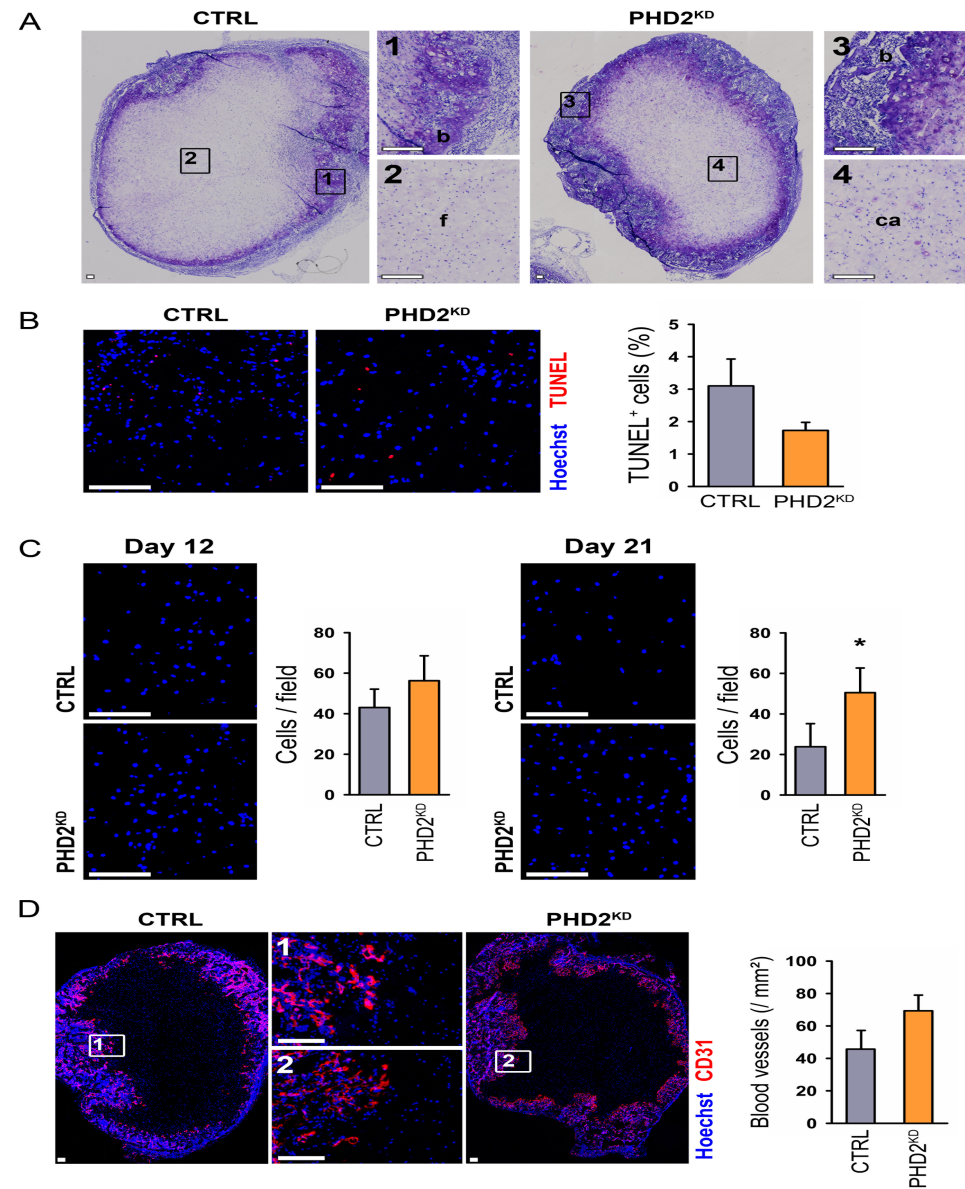
Supplementary figure 4



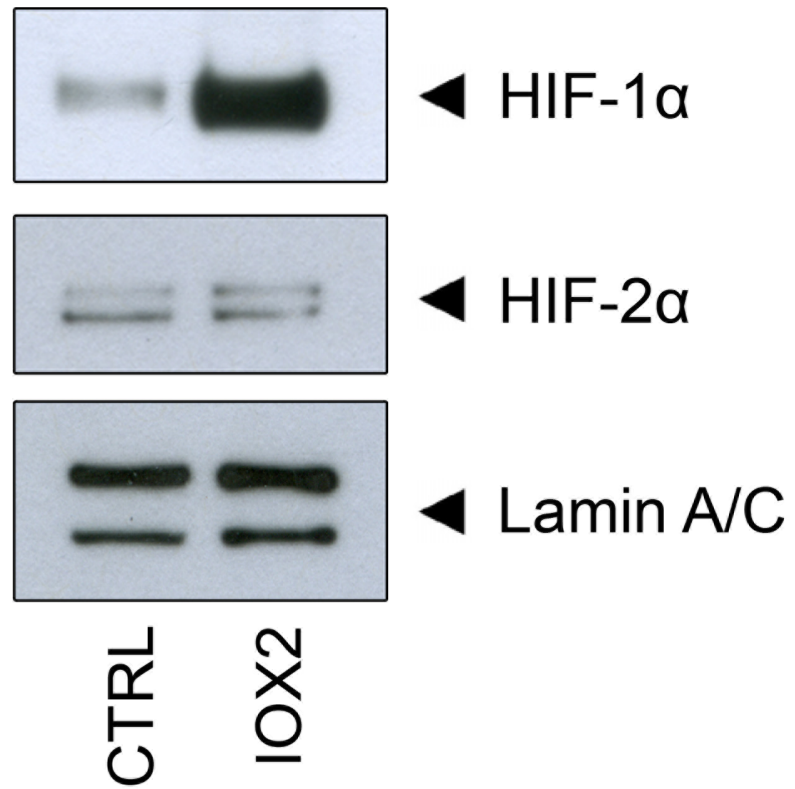
Supplementary figure 5



Supplementary figure 6



Supplementary figure 7



gene	5' primer sequence	3' primer sequence	probe sequence
<i>Hprt</i>	TTATCAGACTGAAGAGCTACTGTAATGATC	TTACCAGTGTCAATTATATCTTCAACAATC	TGAGAGATCATCTCCACCAATAACTTTTATGTCCC
<i>Vegfa(164)</i>	TGCAGGCTGCTGTAACGATG	GAACAAGGCTCACAGTGATTTTCT	TGTCTTTCTTTGGTCTGCATTACATCGG
<i>Phd2</i>	CTGGGCAACTACAGGATAA	CATAGCCTGTTCCGTTGCCT	ACGAAAGCCATGGTTGCTTGT
<i>Sox9</i>	TCTGGAGGCTGCTGAACGA	TCCGTTCTTCACCGACTTCCT	CAGCACAAGAAAGACCACCC
<i>Col2</i>	AGAACATCACCTACCACTGTAAGAACA	TGACGGTCTTGCCCCACTT	CCTTGCTCATCCAGGGCTCCAATG
<i>Acan</i>	GCATGAGAGAGGCGAATGGA	CTGATCTCGTAGCGATCTTTCTTCT	CTGCAATTACCAGCTGCCCTTCACGT
<i>Osx</i>	TGGCGTCCTCTCTGCTTGA	CTAGAGCCGCCAAATTTGCT	AGAAGCTCACTATGGCTC
<i>Ocn</i>	GGCCCTGAGTCTGACAAAGC	GCTCGTCACAAGCAGGGTTAA	ACAGACTCCGGCGCTACCTTGGAGC

Antibody	Supplier	Product No.	Concentration
SOX9	Novus Biologicals (Littleton, CO, USA)	NBP1-85551	1/200
CD31	BD Pharmingen (Franklin Lakes, NJ, USA)	MEC13.3	1/50
TUNEL	Roche Diagnostics GmbH (Mannheim, Germany)	12 156 792 910	Per supplier's protocol
COL2	EMD Millipore (Burlington, MA, USA)	MAB8887	1/200
H3-p	Life Technologies (Ghent, Belgium)	A11010	1/500
BrdU	Abd Serotec (Oxford, United Kingdom)	OBT0030	1/100