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Adequate hypoxia inducible factor 1α signaling is indispensable for bone regeneration

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ABSTRACT

Engineered cell-based constructs are an appealing strategy to treat large skeletal defects. However, transplanted cells are often confronted with an environment that is deprived of oxygen and nutrients. Upon hypoxia, most cell types activate hypoxia-inducible factor 1α (HIF-1α) signaling, but its importance for implanted osteoprogenitor cells during bone regeneration is not elucidated. To this end, we specifically deleted the HIF--1α isoform in periosteal progenitor cells and show that activation of HIF-1α signaling in these cells is critical for bone repair by modulating angiogenic and metabolic processes. Activation of HIF-1α is not only crucial for blood vessel invasion, by enhancing angiogenic growth factor production, but also for periosteal cell survival early after implantation, when blood vessels have not yet invaded the construct. HIF-1α signaling limits oxygen consumption to avoid accumulation of harmful ROS and preserve redox balance, and additionally induces a switch to glycolysis to prevent energetic distress. Altogether, our results indicate that the proangiogenic capacity of implanted periosteal cells is HIF-1α regulated and that metabolic adaptations mediate post-implantation cell survival.

1. Introduction

Regenerative medicine involves the engineering of novel tissue by the use of (adult) stem or progenitor cells to restore damaged organ function. However, grafted cells often encounter an injured tissue that is poorly perfused, rendering them susceptible to lethal damage caused by oxygen and nutrient depletion [1–3]. Cells have however developed mechanisms to resist tissue hypoxia and these are mediated by hypoxia inducible factors (HIFs) that execute the cellular response to low oxygen tensions and HIF prolyl hydroxylases (PHDs) that regulate HIF levels in an oxygen-dependent manner [4,5].

HIF-1 and HIF-2 are heterodimeric transcription factors composed of a constitutively expressed HIF-β subunit and an oxygen-regulated HIF-α subunit. When sufficient oxygen is available, the PHDs mediate the oxygen-dependent hydroxylation of residues in the α-subunit of the HIF protein, rendering it a target for the E3 ubiquitin ligase Von Hippel–Lindau (VHL) and subsequent proteasomal degradation [4,5]. Under hypoxic conditions, the HIF-α/β heterodimer promotes gene transcription intended to restore oxygen delivery by inducing an angiogenic response [6] as well as to adapt cellular energy metabolism [7] to survive oxygen and nutrient depletion.

The vasculature is essential for the transport of oxygen and nutrients to tissues and the removal of waste products. Angiogenesis, or the formation of new blood vessels from pre-existing ones, is a complex process that involves several cell types and signaling pathways. The expression of many of these signaling molecules, including vascular endothelial growth factor (VEGF), is controlled by hypoxia and often mediated by HIF-1α signaling [8,9]. We and others have studied the importance of HIF-dependent VEGF production during skeletal development, homeostasis and pathology [10]. Genetic activation of the HIF signaling pathway in osteoprogenitors or mature osteoblasts resulted in dense, heavily vascularized long bones, associated with increased expression of VEGF [11–14]. On the other hand, inactivation of HIF-1α in mature osteoblasts reduced bone volume and blood vessel number [11,15]. Recent studies have however proposed VEGF-independent effects of HIF-1α in mouse chondrocytes [16] and osteoprogenitors [14], suggesting cell-autonomous regulation of cell function independent of angiogenesis.

The implantation of a cell-based construct in a poorly vascularized environment induces an angiogenic response, mediated by increased production of angiogenic growth factors as a reaction to the local hypoxia. However, blood vessel ingrowth is limited to less than a millimeter per day [17,18], which is insufficient to timely revascularize a tissue of

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clinically relevant size [19]. In order to survive an abrupt drop in oxygen tension, hypoxic cells actively rewire cellular metabolism to maintain energy and redox homeostasis. Indeed, HIF-1α transactivates several genes encoding glucose transporters (GLUTs) and glycolytic enzymes to increase the flux from glucose to lactate [20–23] and stimulates pyruvate dehydrogenase kinase (PDK)-dependent inhibition of the conversion of pyruvate into acetyl-CoA to prevent glucose-derived carbons entering the tricarboxylic acid (TCA) cycle to support oxidative phosphorylation [24,25]. Moreover, hypoxic cells attenuate mitochondrial respiration and increase its efficiency *via* multiple mechanisms, including a switch from cytochrome C oxidase subunit IV isoform 1 (COX4-1) to COX4-2, upregulation of NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4-like 2 (NDUFA4L2) and inhibition of mitochondrial biogenesis [24–28], which is necessary to dampen the production of reactive oxygen species (ROS). Additionally, oxidative stress is further counteracted by a HIF-driven antioxidant response [19,29]. A few studies report that hypoxic preconditioning elicits metabolic adaptations that are beneficial for post-implantation survival of progenitor cells in ischemic tissues [19,30,31], but whether endogenous HIF-1α signaling during bone repair mediates similar cell-autonomous processes remains unexplored.

In this study, we have investigated whether and how HIF-1α is involved in bone regeneration. We report that activation of the HIF signaling pathway stimulates blood vessel ingrowth and bone formation in cell-based constructs, which was manifestly reduced after HIF-1α silencing. Moreover, HIF-1α-dependent changes in mitochondrial function and glycolysis are necessary to support redox and energy homeostasis and are thus critical to ensure cell survival in hypoxia.

2. Materials and methods

2.1. Animals

Hif-1α$^{fl/fl}$ mice (C57BL/6 background) in which exon 2 was flanked by LoxP sites were a kind gift from P. Carmeliet, and were generated as described before [32]. NMRI nu/nu nude mice were purchased from the R. Janvier Breeding Center (Le Genest St. Isle, France). *Hif-1α*$^{fl/fl}$ mice were bred in conventional conditions in our animal housing facility (Proefdierencentrum Leuven, Belgium). Housing and experimental procedures were approved by the Institutional Animal Care and Research Advisory Committee of the KU Leuven.

2.2. Cell culture

2.2.1. Isolation and culture of murine periosteal cells

Murine periosteal cells were isolated from 7 to 9-week-old male mice according to the protocol described previously [33]. Cells were grown in αMEM with 2 mM glutaMAX™-1, containing 1% penicillin/streptomycin and 10% fetal bovine serum (all from Gibco, Life Technologies, Belgium). Upon 80% confluency, cells were trypsinized and reseeded at a 1/3 ratio.

2.2.2. Adenoviral transduction

To silence HIF-1α expression, *Hif-1α*$^{fl/fl}$ periosteal cells were transduced with adenovirus-Cre (Ad-Cre; Gene Transfer Vector Core, University of Iowa, USA) at a multiplicity of infection (MOI) of 500. As a control, cells were treated with Ad-GFP (Gene Transfer Vector Core) or Ad-RR5 (kindly provided by J. Swinnen) at the same dose. After 24 h, virus-containing medium was changed to normal culture medium and 48 h later, cells were used for further experiments.

2.2.3. Interaction of periosteal cells with endothelial cells

A co-culture system was used to study the interaction of periosteal cells and endothelial cells, as described in [33]. Briefly, periosteal cells were seeded and 24 h later human umbilical cord vein endothelial cells (Lonza, Belgium) were added. The co-cultures were grown for 7 days in periosteal growth medium with or without the addition of recombinant soluble fms-related tyrosine kinase-1 (sFlt-1; 100 ng/ml; R&D systems, USA) or anti-murine VEGF-164 antibody ($\alpha VEGF_{164}$; 200 ng/ml; R&D systems). After fixation, the endothelial cells were visualized by staining with a mouse-anti-human CD31 primary antibody (Dako, Denmark) and the TSA Cyanine 3 System (NEN, PerkinElmer, USA).

2.2.4. Osteogenic differentiation

For osteogenic differentiation, periosteal cells were seeded and upon confluency, medium was switched to osteogenic differentiation medium (periosteal cell growth medium supplemented with 50 μM ascorbic acid and 10 mM β-glycerophosphate (both Sigma-Aldrich, Belgium)). After 14 or 21 days, RNA was isolated or cells were stained for alkaline phosphatase or mineral deposition using Alizarin Red, respectively, as described before [34–36].

2.2.5. In vitro cell viability assay

To analyze *in vitro* stress resistance, cells were cultured for 24–72 h in hypoxia (1% oxygen in hypoxic glove box; Coy Lab Products, USA) or in glucose-deprived medium (0.5 mM glucose), or for 4–12 h in the presence of hydrogen peroxide (H_2O_2, 25 μM; Sigma-Aldrich). *In vitro* cell viability was detected with Annexin V propidium iodide (AnxV-PI) staining (Annexin V Alexa Fluor® 488 & propidium iodide Dead Cell Apoptosis kit, Molecular Probes, Life Technologies). Briefly, after harvesting, cells were incubated with 1 × AnxV and 1 μg/ml PI at room temperature for 15 min, analyzed by flow cytometry and the percentage of viable cells ($AnxV^-PI^-$) was calculated using Kaluza™ software.

2.3. Mouse models

To investigate ectopic bone formation and *in vivo* survival, 1×10^6 periosteal cells were seeded onto NuOss scaffolds ($3 \times 3 \times 3$ mm^3; ACE Surgical Supply Co., Inc., Portugal), cultured overnight, and implanted subcutaneously on the back of female 8-week-old NMRI nu/nu mice. For *in vivo* bone formation, scaffolds were retrieved eight weeks after implantation and processed for histology. *In vivo* cell survival was assessed as described in [19]. Briefly, periosteal cells were labeled with CellTracker CM-FDA (Molecular Probes, Life Technologies) prior to seeding. Scaffolds were recovered three days after implantation and cells were collected by enzymatic digest (3 mg/ml collagenase and 4 mg/ml dispase in αMEM with 2 mM glutaMAX™-1, containing 1% penicillin/streptomycin). Cells were subsequently stained with 1× AnxV allophycocyanin (APC) conjugate (Molecular Probes, Life Technologies) and 1 μg/ml PI (Molecular Probes, Life Technologies). Viability of the implanted cells ($CM\text{-}FDA^+AnxV^-PI^-$) was measured using flow cytometry and calculated using Kaluza™ software.

2.4. (Immuno)histochemistry and histomorphometry

Scaffolds implanted for eight weeks were isolated, fixed in 2% paraformaldehyde overnight and decalcified in EDTA for 14 days, before embedding in NEG-50™ frozen section medium (Thermo Fisher Scientific, Belgium) and sectioning with a cryostat at 7 μm. Histochemical (H&E) and immunohistochemical CD31 stainings were described previously [33,35,37,38]. Blood vessel ingrowth three days after implantation was quantified as the amount of $CD31^+$ area relative to the total scaffold. Eight weeks after implantation, the relative number and average size of blood vessels was quantified in the total scaffold. Blood vessels were considered as $CD31^+$ delineated circular structures with an open lumen. Masson's trichrome staining was performed using the Trichrome Stain (Masson) kit (Sigma-Aldrich) according to the