MEET-THE-PROFESSOR: **"HYPOXIA-DRIVEN PATHWAYS IN SKELETAL DEVELOPMENT"**

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Oxygen (O_2) is not only an indispensable metabolic substrate in various enzymatic reactions including mitochondrial respiration, but also a regulatory signal that controls stability and activity of the transcription factors Hypoxia Inducible Factor-1alpha (HIF1) and HIF2, key mediators of the cellular adaptation to low O_2 tension (hypoxia)[1].

Our laboratory has shown that hypoxia-signaling pathways are essential for skeletal development. In our Meet-the-Professor session, I will first present evidence that suppressing mitochondrial respiration is a crucial event downstream of HIF1 during skeletal development (A). Three experimental settings will be discussed, namely fetal growth plate development, formation of synovial joints, and somitogenesis. Next, I will discuss the role of osteoblastic HIF2 in bone mass accrual (B).

Learning Objectives:
1. *Understand the role of oxygen as a regulatory signaling.*
2. *Understand the role of hypoxia signaling pathways in growth plate development, joint formation, somitogenesis, and bone mass accrual*

A. HIF1 and Mitochondria in Skeletal Development
Overall hypothesis: HIF1-dependent suppression of mitochondrial respiration, a critical event in skeletal development.

<u>**Setting 1**</u>**: Fetal growth plate development**
The fetal growth plate is a unique mesenchymal tissue because it is avascular, albeit it requires the angiogenic switch to be replaced by bone. Over the years, we have demonstrated that, consistent with its avascularity, the fetal growth plate has an inner hypoxic region. In addition, we have provided genetic evidence that HIF1 is a survival factor for these hypoxic chondrocytes *in vivo,* as its deficiency causes massive inner cell death in the inner hypoxic region of the fetal growth plate[2-4].
In the attempt to identify the molecular mechanisms that are responsible for the essential and non-redundant role of HIF1 in endochondral bone development, we established that expression of HIF1 in the developing growth plate is important to ensure an adequate number of blood vessels in the perichondrium and a proper accumulation of extracellular matrix in cartilage[5-7]. However, it was surprising to discover that these effects contribute only minimally to HIF1-mediated survival function in chondrocytes[5-7].
During our investigations, we learnt that both viable chondrocytes at the periphery of HIF1 null growth plates of HIF-1 null limb buds are severely more hypoxic than controls[2]. Even more intriguing, we were unable to correct the extreme hypoxia of HIF1 null viable chondrocytes by augmenting delivery of O_2 to the growth plate, through increasing the number of blood vessels in the soft tissue surrounding the mutant cartilaginous molds[5]. The degree of oxygenation of any cell is the net result of O_2 availability and O_2 consumption. Since the severe hypoxia of HIF1 null chondrocytes could not be corrected by increasing O_2 availability, we hypothesized that it had to be the consequence of increased O_2 consumption by the mutant cells. Our hypothesis was in line with the ability of HIF1 to impair mitochondrial respiration and O_2 consumption in a variety of cell types *in vitro.* Furthermore, we hypothesized that increased mitochondrial respiration was a key pathogenetic event in HIF1 null chondrocytes.

To test our hypothesis, since it was not feasible to modulate at once all the downstream targets used by HIF1 to impair mitochondrial respiration, we generated and analyzed mouse models and primary cells that lack HIF1 and Mitochondrial Transcription Factor A (TFAM)[4]. TFAM is not a downstream target of HIF1; however, it regulates expression of numerous enzymes of the mitochondrial respiratory chain. The TFAM conditional knockout mice have been used extensively to inhibit mitochondrial respiration in multiple tissues *in vivo.* Notably, the overall architecture of the fetal growth plate lacking TFAM is remarkably "normal", although hypertrophy is delayed, and limbs are shorter [4]. In addition, loss of TFAM abrogates hypoxia in the fetal growth plate. Those findings indicate that, despite being highly glycolytic, growth plate chondrocytes use mitochondrial respiration as a source of energy in physiologic conditions. Relevant to our hypothesis, loss of TFAM normalizes the shape of HIF1 null bones and prevents to a large extent the cell death of HIF1 null chondrocytes[4]. The rescue of cell death is not secondary to a reduction of ROS levels as ROS are not increased in HIF1 null chondrocytes[4]. More importantly, loss of TFAM corrects the extreme hypoxia of HIF1 null viable chondrocytes [4]. We are currently investigating whether this correction enables survival of HIF1 null chondrocytes with mechanisms that need to be determined and, in light of our current findings, are unrelated to intracellular ATP accumulation.

Setting 2: Synovial joint development

Loss of HIF1 in limb bud mesenchyme delays specification of the interzone and synovial joint development[3]. Those phenotypes are not due to increased cell death[3], and are corrected by simultaneous loss of TFAM (Figure 1)[4].

Setting 3: Somitogenesis *(unpublished data)*

Vertebrae, with their associated musculature and connective tissue, originate from somites during embryonic development. Somites are segmented from the presomitic mesoderm (PSM) and the process is known as somitogenesis. Somitogenesis is controlled by key Notch signals in the PSM that oscillate with a periodicity matching that of somite formation[8, 9]. Consistent with the classical clock-wavefront model, which was proposed over 40 years ago to explain somitogenesis, Notch signals are the segmentation clock of the PSM, where Fgfs and Wnts are wavefront components. The wavefront activity prevents the PSM to respond to the segmentation clock; therefore, segmentation occurs only anteriorly, where the wavefront ends. Hypoxia occurs naturally in developing embryos before the circulatory system is established. However, exacerbation of hypoxia as it may take place during gestation disrupts the oscillatory Notch signals in the PSM and leads to abnormal somitogenesis and spine development. In agreement with those findings, a higher incidence of congenital scoliosis has been reported in human communities who live at high altitude. Spondylocostal Dysostosis (SCDO) is a human disease characterized by severe vertebral malformations and is caused by homozygous loss-of-function mutations (LOF) of components of the Notch signaling pathway [10]. Mice carrying similar homozygous mutations phenocopy the human disease [10]. Heterozygous Notch LOF mutations cause the more modest, although more frequent, human defect of congenital scoliosis (CS), which is also phenocopied in heterozygous mouse mutants[10]. Those
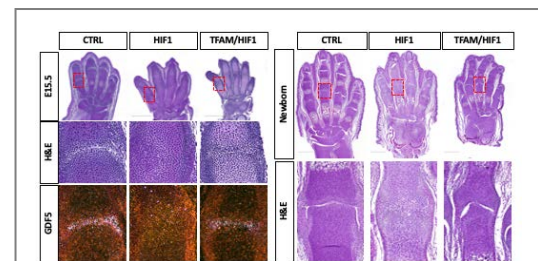

Figure 1: On the left, H&E staining and *in situ* hybridization analysis for *Gdf5* mRNA performed on histological sections of autopods isolated from E15.5 CTRL, HIF1 and TFAM/HIF1 mice, respectively. On the right, H&E staining on histological sections of autopods isolated from newborn CTRL, HIF1 and TFAM/HIF1 mice, respectively.
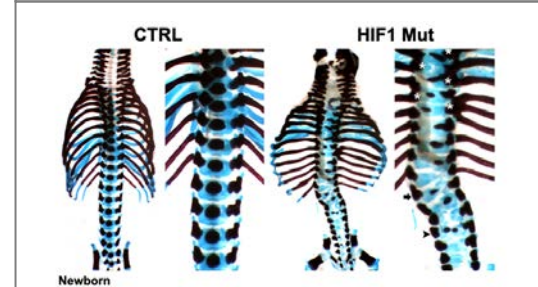

Figure 2: Congenital Scoliosis in TCre;HIF1[flox/null] mutants (HIF1 mutants). Alcian blue and Alizarin Red whole mount staining at birth of control (CTRL) and HIF1 mutants. Black arrows: hemi-vertebrae. Black arrowheads: butterfly vertebrae. White stars: fused ribs at the posterior end. Fused ribs are another typical component of the SCDO/CS phenotype.

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mouse phenotypes are worsened by gestational hypoxia [10]. The role of HIF1 in somitogenesis in either physiological of components of the Notch signaling pathway. Mice carrying similar homozygous mutations

phenocopy the human disease. Heterozygous Notch LOF mutations cause the more modest, although more frequent, human defect of congenital scoliosis (CS), which is also phenocopied in heterozygous mouse mutants. Those mouse phenotypes are worsened by gestational hypoxia. The role of HIF1 in somitogenesis in either physiological conditions or during gestational hypoxia has not been addressed. To fill this gap of knowledge, we conditionally inactivated HIF1 in the PSM using TCre transgenic mice. TCre;HIF1^flox/null (HIF1 Mut) mutant mice displayed an abnormal curvature of the spine with multiple vertebral malformations reminiscent of SCDO/CS (Figure 2). Moreover, whole mount *in situ* hybridization analysis performed on E9.5 embryos (20-25 somites stage) demonstrated that both segmentation of the PSM and expression of Notch signals at this site were severely altered in HIF1 mutants (Figure 3, Figure 4). The above phenotype was not the consequence of cell death (data not shown). This latter finding is particularly relevant as HIF1 is a crucial survival factor for growth plate chondrocytes in the context of endochondral bone development. Taken together, our findings indicated that loss of HIF1 in the PSM causes spine abnormalities by impairing early stages of somitogenesis and without affecting survival of PSM cells.

It is well established that hypoxia increases HIF transcriptional activity; therefore, we were intrigued by the observation that gestational hypoxia and *loss* of HIF1 in the PSM alter somitogenesis in a similar manner. We hypothesized that in both conditions the PSM experiences an increase in intracellular hypoxia for the reasons detailed below. As also discussed above, HIF1 reprograms glycolytic metabolism by promoting glycolysis and lactate fermentation and impairing mitochondrial respiration. Hence, its loss increases mitochondrial respiration, mitochondrial O_2 consumption, and intracellular hypoxia**.** In addition, HIF1 regulates the formation of blood vessel networks through the control of angiogenic factors. Therefore, in a variety of tissues, HIF1 loss decreases the number of local blood vessels and, as a result, O_2 availability.

Considering all this body of knowledge, the current model predicts that loss of HIF1 in the PSM would increase intracellular hypoxia by augmenting O_2 consumption and/or reducing O_2 availability, whereas gestational hypoxia likely augments intracellular hypoxia by reducing O_2 availability to the cells of the PSM. In agreement with our hypothesis, using the hypoxia marker EF5, we confirmed that both loss of HIF1 and gestational hypoxia led to an increase in intracellular hypoxia**,** which was milder in the case of gestational hypoxia most likely because hypoxia-dependent activation of HIF1 was in part compensating for the reduced O_2 availability (Figure 5 and data not
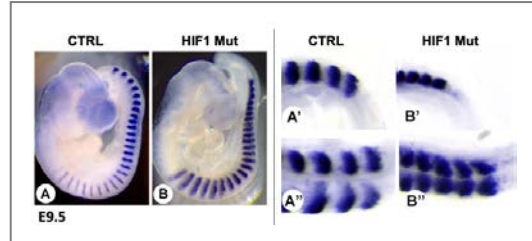

Figure 3: Impaired somitogenesis in HIF1 mutant embryos**.** Whole mount in situ hybridization of E9.5 embryos with Uncx4.1 riboprobe. Magnified views of the last four somites (A' and B'), and their dorsal appearance (A'' and B'') are shown.
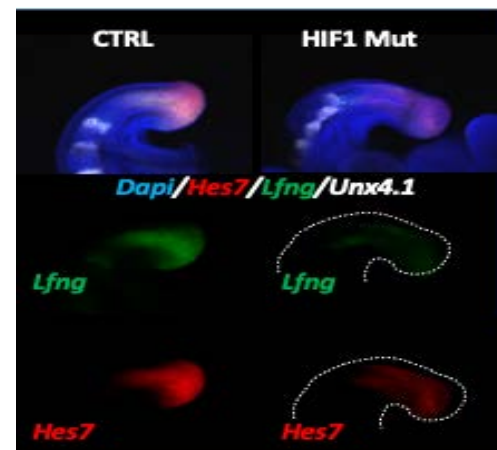

Figure 4: Decreased Notch signaling in the PSM of E9.5 HIF1 mutants. Hybridization Chain Reaction analysis.
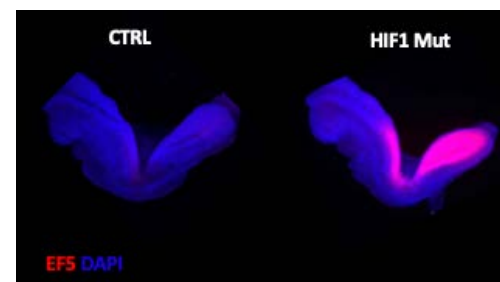

Figure 5: Increased EF5 signal in the PSM of E8.5 HIF1 embryos. CTRL=Control. Of note, at a more prolonged exposure time, EF5 signaling could be detected also in CTRL embryos (data not shown).

shown). Furthermore, exciting preliminary data suggest that haploinsufficiency for TFAM is sufficient to significantly ameliorate the spine defects observed in HIF1 mutants (data not shown). Those findings are in line with what we reported in the developing growth plate and in the digital autopod, namely HIF1 is a differentiation and survival factor by, at least in part, suppressing mitochondrial respiration and thus mitochondrial O_2 consumption.

Our current working model is that both loss of HIF1 in the PSM and gestational hypoxia increase intracellular hypoxia at this site, and this in turn dysregulates the Notch signaling pathway in the PSM and alters early stages of somitogenesis.

At the end of part A, we will discuss the following questions:
1. *How does loss of TFAM delay growth plate hypertrophy?*
2. *How does loss of TFAM enable survival of growth plate chondrocytes, normal joint development, and normal somitogenesis, in absence of HIF1?*

B. HIF2 and Bone Mass Accrual

We recently conditionally deleted HIF2 in mesenchymal progenitors and analyzed their bone phenotype to unveil the physiological functions of HIF2 in osteoblastic cells and establish whether HIF2 is necessary for the control of bone mass accrual[11]. For this purpose, we crossed PRX1-Cre transgenic mice with HIF2[f/f] mice to generate PRX-HIF2[f/f] mutant and PRX-HIF2[f/+] and HIF2[f/f] control littermates. Of note, we had previously established that PRX1-HIF2[f/f] mutants are viable and, prenatally, display only a modest and transient growth plate phenotype, which fully resolves postnatally[11]. MicroCT analysis revealed a significant increase in both the trabecular and cortical bone mass of mutants when compared to controls. Static histomorphometry analysis of trabecular bone confirmed the microCT data[11]. The number of osteoblasts over bone surface (N.Ob/BS), the mineral apposition rate (MAR) and the bone formation rate over bone surface (B FR/BS) were all significantly augmented in mutant bones[11].

Lastly, the osteoclast number over tissue volume (Oc.Nb./TV) was not different between HIF2[f/f], PRX-HIF2[f/+] and PRX-HIF2[f/f] specimens. Conversely, the osteoclast number over bone surface (Oc.Nb./BS) was modestly reduced in PRX-HIF2[f/f] bones in comparison to HIF2[f/f] controls as the result of the increased bone surface in PRX-HIF2[f/f] specimens secondary to the higher bone formation rates in mutants[11].

Our findings constitute a paradigm shift, as activation of the hypoxia signaling pathway has traditionally been associated with increased bone formation through HIF1**.** Inhibiting HIF2 could thus represent a therapeutic approach for the treatment of the low bone mass observed in chronic diseases, osteoporosis, or aging.

At the end of part B, we will discuss the following question:
How does loss of HIF2 in PRX1 lineage cells increase osteoblast number/activity?

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