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ATF4 AND HIF-1α IN BONE: AN INTRIGUING RELATIONSHIP

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As occurs in any other vascularized organ, one of the essential functions of blood vessels in bone is to be a source of O_2, nutrients, hormones and growth factors [(1)]. However, additional roles for bone blood vessels are emerging. For example, osteoblast precursors have been recently identified within their wall [(2,3)], which suggests that blood vessels are a source of osteoprogenitors. Moreover, it has been shown that endothelial cells are specialized niches for hematopoietic stem cells [(4,5)]. In light of these findings, identification of the molecular and cellular mechanisms controlling angiogenesis in bone is essential in order to reach a full understanding of how bone and bone marrow development and homeostasis are regulated.

In this issue of JBMR [(6)], Zhu and colleagues report that activating transcription factor-4 (ATF4) promotes angiogenesis in bone. This effect is associated with an increased production of Vascular Endothelial Growth Factor - A (VEGF) by osteoblasts, and with stabilization of the hypoxia-inducible factor 1α (HIF-1α) in the same cells [(6)]. It has been know for quite some time that ATF4 controls bone homeostasis by stimulating both osteoblastogenesis and osteoclastogenesis [(7-10)]: Zhu and colleagues now add a new element to the picture by showing that mice lacking ATF4 have a reduced number of blood vessels in bone [(6)]. The importance of their study is two-fold: first, it provides convincing evidence that ATF4 regulates bone angiogenesis *in vivo* and *ex-vivo*; second, it shows that ATF4 contributes to stabilize HIF-1α protein in hypoxic-like conditions.

The vast majority of adult normal tissues functions at oxygen (O_2) levels between 2% and 9%, with ambient air at 21% O_2 [(11)]. Cartilage, bone marrow, kidney medulla and thymus, on the other hand, can exist at 1% O_2 or lower [(11)] . When O2 tension goes below 2%, this condition is considered moderate hypoxia. When O_2 tension goes below 0.5%, hypoxia is considered severe. Hypoxia-inducible factor-1 (HIF-1), a ubiquitously expressed transcription factor, is a major regulator of cellular adaptation to hypoxia [(12-18)]. It is a heterodimeric DNA-binding complex that consists of two proteins, HIF-1α and HIF-1β [(19)]. HIF-1α is activated when O_2 levels drop below 5% [(20-25)]. On the other hand, HIF-1β is non-oxygen responsive. HIF-1α does not directly sense variations of O_2 tension [(26)]; a class

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of 2-oxoglutarate-dependent and Fe^{2+}-dependent prolyl-4-hydroxylases (PHDs) are the O_2 sensors [20]. PHDs hydroxylate two prolyl residues (P402 and P564) in the HIF-1α region referred to as the O_2-dependent degradation domain [27]. This modification occurs in normoxic conditions and mediates the binding of the von Hippel-Lindau tumor suppressor protein (pVHL), which is an E3 ubiquitin ligase, to HIF-1α HIF-1αis then marked with polyubiquitin chains and targeted for degradation by the proteosome. Under hypoxic conditions, the activity of the PHDs is impaired and proline hydroxylation cannot occur. As a result, HIF-1α protein accumulates and this initiates a multi-step pathway that includes nuclear translocation of HIF-1α, dimerization with its partner HIF-1β, recruitment of transcriptional co-activators, and binding to hypoxia-responsive elements within the promoters of hypoxia-responsive genes [28].

HIF-2α, another member of the family, has been recently identified and characterized [17]. Similarly to HIF-1α, HIF-2α is degraded by the proteasome in normoxia, whereas it is stabilized in hypoxia [17,18]. VEGF is one of the direct downstream targets of both HIF-1α and HIF-2α[17,29].

Despite its high degree of vascularization, a gradient of oxygenation is present in the bone marrow, and the endosteal surface of cortical bone is among the most hypoxic areas as revealed by staining with the marker of hypoxia pimonidazole [30-32]. This gradient of oxygenation within the bone marrow is most likely created by the high degree of bone marrow cellularity, the high levels of O_2 consumption by hematopoietic cells, and the slow flow in the bone marrow sinusoids [32,33].

Several laboratories are currently investigating how bone cells and bone blood vessels relate to each other. In particular, it has been recently shown that either osteoblastic stabilization HIFs or osteoblastic overexpression of VEGF, one of the most powerful proangiogenic growth factors, leads to a dramatic increase of trabecular bone and to a significant augmentation of the number and/or volume of bone marrow blood vessels [1,18,29,34-36]. These findings have prompted researchers to conclude that an osteogenesis-angiogenesis coupling is likely to exist and to play a role in bone development and homeostasis [18]. ATF4 has now been added to the list of transcription factors that control bone angiogenesis and, possibly, this osteogenesis-angiogenesis coupling [6]. Notably, Zhu and colleagues show in their paper that ATF4 not only is sufficient to drive angiogenesis, but is also necessary to ensure a normal number of blood vessels in bone. Moreover, they propose, though they do not experimentally prove, that osteoblastic VEGF downstream of osteoblastic HIF-1α is the key mediator of this novel function of ATF4 [6]. Increased release of VEGF from the matrix upon activation of bone resorption by osteoclasts could be an additional contributing factor [6].

Taken together, these are interesting and intriguing findings. However, since Zhu and colleagues have used for their study the global knockout of ATF4 rather than its conditional knockout in cells of the osteoblast lineage, at this stage we cannot conclude with absolute certainty that osteoblastic ATF4 is indeed the main responsible of the angiogenic phenotype observed in mutant mice lacking ATF4. Along these lines, the activation of osteoclasts and

the release of VEGF from the bone matrix occurring upon universal loss of ATF4 are probably both osteoblast–dependent and osteoblast-independent effects [6].

The notion of an osteogenesis-angiogenesis coupling, which would be essential not only in bone development and bone repair but also in bone homeostasis, is potentially very important. However, numerous issues still need to be addressed and resolved. Undoubtedly, all the studies presented and discussed to this end strongly suggest the existence of a correlation between these two events, osteogenesis and angiogenesis [1,3,18,29,34,35]. Nonetheless, while it has been convincingly established that stabilization of HIFs in cells of the osteoblast lineage or overexpression of VEGF in the same cells causes a dramatic increase of bone mass in the trabecular compartment as well as an augmentation of bone vascularity[1,18,29,34-36], it has never been experimentally tested whether the increased number of blood vessels is an essential prerequisite for the high bone mass phenotype observed in these models. The same consideration applies to the global knockout of ATF4: loss of ATF4 impairs both bone mass and bone angiogenesis [6], but to this end we cannot unequivocally conclude that the decreased number of blood vessels is the main factor contributing to the low bone mass phenotype observed in ATF4 null bones. Supporting our concern, it has been recently shown that osteoblastic VEGF controls osteoblastogenesis mainly through an intracrine mechanism, rather than by regulating number of blood vessels in the bone marrow [37].

Moreover, it is well established that osteoblasts regulate bone marrow angiogenesis [18] and that VEGF is a very powerful proangiogenic factor [38], but the involvement of additional cytokines and/or growth factors produced by osteoblasts cannot be excluded.

In addition, the notion that also osteoclasts could be angiogenic cells by either producing angiogenic factors or by releasing them from the bone matrix has recently emerged [39].

Last, both ATF4 and HIFs are likely to control bone homeostasis with a variety of mechanisms that go beyond angiogenesis.

In this regard, HIF-1α is a crucial regulator of glucose metabolism. In aerobic conditions, glucose is converted to pyruvate in the cytoplasm. Pyruvate then enters the tricarboxylic acid (TCA) cycle and oxidative phosphorylation takes place in the mitochondria [40]. Louis Pasteur was the first to record that O_2 -deprived cells convert more glucose to lactate than cells in normoxic cultures. This is the so-called “Pasteur effect”. Induction of the Pasteur effect depends on HIF-1α which up regulates glucose transporters, glycolytic enzymes, and the enzyme lactic dehydrogenase [41-43]. Moreover, HIF-1α inhibits mitochondrial oxidative phosphorylation, at least in part, by augmenting levels of pyruvate dehydrogenase kinase, an enzyme that phosphorylates and inhibits pyruvate dehydrogenase and thus conversion of pyruvate into acetyl CoA [44,45]. By inhibiting the entry of pyruvate into the mitochondria, HIF-1α attenuates not only mitochondrial respiration, but also diminishes ROS production in hypoxic cells [46]. Notably, it has been recently reported that both activation of non-oxidative glycolysis and accumulation ROS significantly affect bone homeostasis [47-49], which implies that HIFs could indeed control bone mass at least in part by modulating glycolysis and oxidative phosphorylation in osteoblasts. With respect to ATF4, it has

already been unequivocally shown that ATF4 in osteoblasts activates both gene transcription and amino acid import, and both functions are crucially important for ATF4-dependent regulation of osteoblastogenesis [7-9].

Notably, ATF4 is a critical mediator of the endoplasmic reticulum stress response, particularly in hypoxic conditions [50]. Hypoxia increases ATF4 levels in a HIF-independent manner [50], and, at least in part, through inhibition of PHD3 activity [51]. These findings indicate that the PHD-oxygen sensing recruits both HIFs and ATF4. Zhu's paper closes the loop between hypoxia, ATF4 and HIF-1α by showing that in cells of the osteoblast lineage, ATF4 is essential for proper stabilization of HIF-1α in hypoxia [6]. The details of this interaction are not yet clear, though they may again involve the oxygen sensing machinery. In any event, the finding that HIF-1α and ATF4 appear to converge on common O_2/nutrient sensing pathways in higher animals is consistent with the emergence of these two transcriptional pathways at a similar time in metazoan evolution [52,53] Interestingly, also RUNX2, another regulator of VEGF expression [54], has been reported to interact with HIF-1α and control its stability [55].

All in all, an interesting feedback mechanism has been established in which hypoxia increases ATF4 stability in a HIF-independent fashion, and this leads to further stabilization of HIF-1α with modalities that need to be further elucidated. At this stage, it is unknown whether this novel loop occurs only in osteoblastic cells, or whether indeed is a more general mechanism of HIF-1α regulation also present in other cell types. Moreover, it is unknown whether stabilization of HIF-2α in hypoxia does require interaction with ATF4.

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