FoxO1: A Molecule for All Seasons

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ABSTRACT
The FoxO family of forkhead transcription factors is at the crossroads of many signal transduction pathways that are evolutionarily conserved. Such pathways have been co-opted in differentiated tissues for a variety of vital and specialized functions, such as differentiation, proliferation, and survival in cells as diverse as adipocytes, hepatocytes, β-cells, myoblasts, thymocytes, and cancer cells. FoxO metabolic functions are relevant to glucose metabolism, tumor suppression, hematopoiesis, angiogenesis, and antioxidant defense. Among the FoxO isoforms, FoxO1 is a main target of insulin signaling and regulates metabolic homeostasis and organismal survival at many different levels. FoxO1 entered into the field of skeletal biology by a property that is unique among its functions in other organs. With the osteoblast as its target cell, FoxO1 not only acts on it to regulate bone homeostasis but also through it as a transcriptional modulator of the endocrine function of the skeleton in regulating glucose metabolism. Through its direct skeletal actions, FoxO1 promotes osteoblast proliferation by maintaining protein synthesis and redox balance. Through its endocrine actions on target tissues of insulin, FoxO1 acts by way of osteocalcin to suppress glucose production by pancreatic β-cells and hepatocytes and to decrease insulin production and sensitivity. These two parallel but opposing actions, one in favor of the skeleton and the other in disadvantage of glucose-regulating tissues, may signify an adaptive mechanism that integrates responses between different organs and is beneficial for whole-body physiology during stress and aging. © 2011 American Society for Bone and Mineral Research.

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Introduction

Model organisms have become an effective tool for studying mammalian biology in part because they provide a more simplified system whereupon the function of several molecules can be examined. When the biology of model organisms and mammals is closely compared, a few molecules are likely to be identified that retain their function, their sequence homology, and their cellular targets across species. These highly conserved molecules are likely to be very important. The FoxO family of ubiquitous transcription factors, which belong to the forkhead family of proteins, illustrates this point well. FoxOs and their signal transduction pathways are highly conserved across diverse species, from C. elegans to mammals. Additionally, over evolutionary space, FoxO members have been co-opted by specialized tissues for a variety of key functions. This multifunctionality extends to differentiation, proliferation, and survival in cells as diverse as adipocytes, hepatocytes, β-pancreatic cells, myoblasts, thymocytes, and cancer cells. Among the many biological functions they serve, FoxO proteins are known best for three defining properties: survival, by means of resistance to oxidative stress; glucose metabolism, a property specific to FoxO1; and suppression of tumorigenesis, a property also mainly regulated by FoxO1. Balancing the multiple avenues of FoxO activity are several opposing signaling pathways that regulate their localization to the nucleus, where they are active. Among the endogenous inhibitors of FoxOs, growth factors, such as insulin and insulin-like growth factors, signal phosphorylation of FoxOs via phosphatidylinositol 3-kinase and Akt kinases, favoring their retention in the cytoplasm. On the other hand, among the endogenous enhancers, kinases such as JNK or the mammalian ortholog of the St20-like protein kinase (MST1) stimulate phosphorylation of FoxO in response to oxidative stress at other sites and lead to translocation of FoxO into the nucleus. Once in the nucleus, FoxOs define cell fate by a variety of functions, such as transactivating specific cyclins, cyclin-dependent kinase inhibitors, DNA repair, apoptosis-control genes, and antioxidant enzymes.

There are four FoxO molecules: FoxO1, FoxO3, FoxO4, and FoxO6. They are all encoded by different genes, with FoxO6 being structurally and functionally distinct from the other three isoforms, which are closely related to each other. Among them, FoxO1 is particularly noteworthy in that it regulates the most diverse array of the FoxO’s known biological activities, including organ growth, insulin action, tumorigenesis, and angiogenesis. This review focuses on FoxO1 and its emerging set of functions in and through bone cells.
FoxO1 actions on bone

Interest in the role of FoxO1 in bone physiology originates from two observations. The first one is the premise that osteoporosis is, at least in part, a disease of aging. It is reasonable to hypothesize that similar mechanisms governing the general aging process may also apply to the pathogenesis of age-associated bone loss. Among the many pathways implicated in aging, oxidative stress is most consistently observed, both in aging per se and in age-associated human diseases. Similar to other age-related diseases, osteoporosis is associated with increased levels of oxidative stress in osteoblasts, suggesting that they may be a critical component of the pathophysiology of bone loss. To test this hypothesis without expanding to too many different proteins, one should look for candidate transcription factors that regulate the expression of many genes involved in antioxidant defense. Indeed, FoxOs are one of the two major components that cells use to counteract the adverse effects of oxidative stress. This response involves transcriptional activation of FoxOs and subsequent upregulation of the expression of three main FoxO targets: the mitochondrial enzyme superoxide dismutase 2 (SOD2), which converts hydroxyl radicals to H₂O₂; the peroxidase catalase, which converts H₂O₂ to water; and GADD45, the growth-arrest and DNA-damage-inducible protein. It is through their role as orchestrators of antioxidant defense signaling mechanisms that FoxOs were identified as regulators of skeletal homeostasis.

This hypothesis was tested, as it should be, genetically and in vivo. Through a series of cell-specific gene deletion and molecular analyses, it was shown that among the three FoxO proteins, FoxO1 is the main regulator of redox balance and function in osteoblasts and the only one that overtly controls bone mass. Deletion of FoxO1 specifically from osteoblasts and osteoblast progenitors decreases osteoblast numbers, bone formation rate, and bone volume. In this deletion model, markers of osteoblast function were much more impressively compromised than the concomitant increase in bone resorption indices. Skeletal deterioration in this osteoblast-specific FoxO1 knockout model was correlated with increased oxidative stress levels specifically in cells of the osteoblastic lineage from which FoxO1 was deleted, as evidenced by elevation of both reactive oxygen species (ROS) and lipid peroxidation products. Oxidative stress was secondary to a suppression of antioxidant defense mechanisms, as indicated by a decrease in the activity of SOD2 and the levels of glutathione, a protein that in its reduced form scavenges free radicals and detoxifies cells. Consistent with these observations, conditional deletion of all three FoxO isoforms, FoxO1, FoxO3, and FoxO4, in all cell types decreased osteoblast numbers and increased oxidative stress in bone. The antiresorptive effect of FoxO1 may be related to the ability of FoxOs to interact with β-catenin. It has been shown that β-catenin suppresses osteoclast formation by increasing the expression of osteoprotegerin by osteoblasts and FoxOs interact with β-catenin to divert it from TCF- to FoxO-mediated transcription.

Although supportive of a role for FoxO1 in bone metabolism, these observations were to a large extent, phenomenological. To demonstrate a direct, mechanistic link between FoxO1-regulated skeletal metabolism and oxidative stress, two questions had to be addressed. First, was the increase in oxidative stress in osteoblasts the reason for compromised osteoblast numbers? If this could be demonstrated, the second question would then be: What was the cause of the increase in oxidative stress and by what means did it suppress osteoblast numbers? To answer the first question, oxidative stress levels in osteoblasts were normalized by treating mice lacking FoxO1 in osteoblasts with the antioxidant N-acetyl l-cysteine (NAC). NAC rescued the stress-associated abnormalities of mutant mice, including osteoblast numbers, bone formation rate, and bone volume. This experiment proved that the increased oxidative stress in osteoblasts is directly responsible for the low-bone-formation phenotype. It also defined oxidative stress, in principle, as a negative regulator of bone mass.

The answer to the second question, which was directed at elucidating the cause of the increase in oxidative stress, took an approach that originated from observations indicating that protein synthesis by osteoblasts is compromised following FoxO1 deletion. Unraveling the mechanism of these events, FoxO1 was shown to regulate amino acid import and thus protein synthesis in osteoblasts. This function results from the physical interaction of FoxO1 with ATF4, a transcription factor regulating amino acid import and collagen synthesis by osteoblasts (Fig. 1). The reduction in protein synthesis was the causative factor for the reduction in the levels of antioxidant defense molecules such as glutathione and thus the increase in oxidative stress levels.

Explaining how these events could confer the deleterious signal of oxidative stress to osteoblasts, it was found that in osteoblasts, FoxO1 utilizes a previously unrecognized mechanism to organize antioxidant responses and maintain osteoblast proliferation (Fig. 1). This mechanism involves inhibition of a p53-dependent signaling pathway that mediates ROS-induced antiproliferative actions and early senescence. Two proteins, the products of p19Arf and p16, activate a p53 pathway that leads to antiproliferative effects. FoxO1 controls osteoblast proliferation by promoting protein synthesis through interaction with ATF4 and downstream regulation of a stress-dependent pathway that inhibits p53 signaling. These results established that FoxO1 is a transcriptional determinant of redox balance and, thus, a purveyor of normal function of osteoblasts.

FoxO1 actions through bone on other systems

FoxO1 regulates whole-organism physiology by being involved in many key metabolic functions. From glucose metabolism to tumor suppression, hematopoiesis, angiogenesis, and antioxidant defense, FoxO1 regulates metabolic homeostasis and organism survival at many different levels. However, its skeletal actions reveal a unique characteristic: it affects other organs through its skeletal actions. Indeed, FoxO1 is a transcription factor that orchestrates the endocrine function of the skeleton in regulating energy metabolism. The metabolic actions of osteoblast-expressed FoxO1 are likely to be mediated, at least in part, by osteocalcin (see below). As shown by several laboratories, osteocalcin, in its uncarboxylated state, favors β-cell proliferation, insulin secretion, and sensitivity.
Osteocalcin carboxylation is promoted by protein tyrosine phosphatase, the product of Esp, and a protein that inhibits insulin signaling in osteoblasts. On the other hand, insulin signaling in osteoblasts promotes bone resorption in a FoxO1-dependent manner and as a result induces the acidification of the bone extracellular matrix. The acidic environment generated during osteoclastic bone resorption in turn promotes osteocalcin decarboxylation.

Bearing in mind this novel endocrine function of the skeleton, it was unavoidable to correlate it with the most well known and more extensively characterized function of FoxO1: its role as the major transcriptional mediator of insulin signaling in all insulin target tissues. FoxO1 suppresses pancreatic β-cell proliferation and function and is a negative regulator of insulin sensitivity in β-cells, hepatocytes, and adipocytes. FoxO1 haploinsufficiency partially reinstates the reduced β-cell proliferation observed in Insulin receptor substrate 2 (Irs2)–deficient mice as well as in mice with β-cell-specific deletion of Pdk1, a PI3K-dependent protein kinase that is important in maintenance of β-cell mass. In the hepatocyte, FoxO1 promotes gluconeogenesis by acting in concert with the PPARγ coactivator PGC1α to stimulate the expression of glucose-6-phosphatase (G6pase) and phosphoenolpyruvate kinase 1 (Ppck1). Thus, FoxO1 controls at least three important aspects of glucose metabolism: insulin production, insulin sensitivity, and hepatic glucose production. How these well-known functions of FoxO1 relate to its role in osteoblastic function remained to be elucidated. Thus, it was reasonable to examine whether FoxO1 regulates glucose metabolism, in part, through its osteoblastic expression.

In support of a putative relationship between FoxO1 in the osteoblast and FoxO1 actions in the classical glucose-regulating organs, mice lacking FoxO1 in osteoblasts show reduced blood glucose levels in both the fasting and the fed state. The cause of the hypoglycemia is 2-fold: an increase in insulin production as well as an increase in insulin sensitivity. The increase in plasma insulin levels is the result of increased β-cell proliferation, higher islet cell numbers, greater islet size, and total β-cell mass in mice with osteoblast-specific deletion of FoxO1. Hyperinsulinemia is consistent with a suppression in gluconeogenesis and is independent of any contributions from counter-regulatory hormones with anti-insulinemic activity, such as glucagon or growth hormone. It is also independent of any potential actions of FoxO1 in the hypothalamus, where it has been shown to regulate food intake and peripheral metabolism by interacting with leptin signaling.

Experiments in which FoxO1 was deleted in osteoblasts led to better disposal of glucose load and a marked improvement in glucose tolerance. Consistent with higher insulin levels and greater insulin sensitivity, the expression of several insulin target genes in muscle, liver, and white adipose tissue was altered. Similarly, liver fat content and mitochondrial activity in muscle were increased, indicating that FoxO1, through its expression in osteoblasts, inhibits insulin sensitivity in liver and muscle. Despite...
hyperinsulinemia and improved insulin sensitivity, mice with osteoblast-specific deletion of FoxO1 showed reduced gonadal fat pad weight. Explaining, at least in part, the low gonadal fat weight, both energy expenditure and activity levels were increased, whereas energy intake was not affected. Taken together, these abnormalities are reminiscent of observations in Esp-deficient mice, a model of osteocalcin gain of function.\textsuperscript{[28]}

The observations linking FoxO1 and osteocalcin to energy metabolism raised the possibility that these actions of FoxO1 and osteocalcin were directly related to each other. The notion of a functional relationship between FoxO1 and osteocalcin was supported by comparisons of the metabolic phenotypes of mice lacking FoxO1 in osteoblasts with the metabolic phenotypes of gain-of-function and loss-of-function mouse models of osteocalcin activity. It was easily observed that whereas the metabolic phenotype of FoxO1 mutant mice mirrored that of mice lacking Esp and showed increased osteocalcin activity, it was exactly opposite to the phenotype of mice lacking osteocalcin. In agreement with these observations, Esp expression in bone was decreased and osteocalcin activity, measured by the percentage of its uncarboxylated form in the serum, was increased in mice lacking FoxO1 in osteoblasts. These initial observations suggested a link between FoxO1 expression and osteocalcin activity. Proving this notion genetically, heterozygous mice lacking a single allele of FoxO1 and Esp showed improved insulin sensitivity and glucose tolerance.\textsuperscript{[27]} Likewise, removal of one allele of osteocalcin from mice lacking FoxO1 from osteoblasts corrected the metabolic phenotype of improved glucose tolerance and insulin sensitivity that results from osteoblast-specific inactivation of FoxO1. Collectively, these observations showed that FoxO1 can control glucose metabolism through osteoblasts, at least in part, by regulating the activity of osteocalcin (Fig. 1).

FoxO1 in whole-organism biology

In the past few years, the so-called “hormesis hypothesis” has emerged to explain how multiple, disparate stimuli can lead to the same longevity response in a variety of species.\textsuperscript{[45–51]} The hypothesis states that a variety of mildly stressful conditions provoke a survival response within the organism, helping it to survive adversity by altering metabolism and increasing the organism’s defenses against causes of aging (such as mild heat, increased salt, low amino acids, or low glucose). Eventually, all these stimuli promote cellular and organ function. In analogy with this hypothesis, the skeleton is a dynamic organ that is continually subjected to several stress stimuli: biomechanical stress; oxidative and metabolic stress induced by hormonal fluctuations during pregnancy, menstrual cycle, and eventually menopause; and oxidative and metabolic stress related to a variety of endocrinological and cellular changes that characterize growth, adulthood, and aging. It is possible that “skeletal hormesis” — beneficial actions resulting from the response of bone to continuous, low-intensity physiologic stress signals from any of the above stressors, is essential for normal bone cell function, preservation of bone mass, and skeletal regeneration.

This hypothesis is also applied within the context of aging and is tightly linked to oxidative stress. There is significant evidence supporting the notion that free radicals play an important role in aging, either as “damaging” molecules or as signaling molecules. Age-associated oxidative injuries induced by free radicals, higher susceptibility to oxidative stress, genetic manipulations that alter oxidative resistance, and the antiaging effect of caloric restriction are only a few examples that implicate oxidative stress in the aging process and in the development of aging-associated diseases. Yet the mechanisms and mediators that implement the effects of oxidative stress in different tissues remain unknown. The identification of FoxO1 as a crucial mediator of ROS signaling in osteoblasts has led to the delineation of the molecular events that mediate the effects of oxidative stress on osteoblast proliferation. FoxO1 confers these actions in two ways. First, it is required to prevent the adverse effects of elevated levels of reactive oxygen species, which can damage proteins, lipids, and DNA, eventually compromising osteoblast function. Second, its activation under low physiological stress levels is advantageous because it stimulates defense signaling mechanisms that maintain and even prolong osteoblast and skeletal functionality.

Perspective

FoxO1 is an important mediator of insulin’s effects on pancreatic and hepatic glucose metabolism. It is also a transcriptional target of insulin signaling in osteoblasts.\textsuperscript{[29]} It was very recently shown that the insulin receptor is expressed in osteoblasts and that insulin acts through it to regulate osteoblast function and glucose metabolism.\textsuperscript{[29,30]} Insulin action in osteoblasts regulates insulin secretion and sensitivity by inducing osteoclastic resorption, which in turns leads to decarboxylation and activation of osteocalcin.\textsuperscript{[29]} Why would FoxO1 reproduce the same functions through its expression in bone, pancreas, and liver? It is now increasingly appreciated that abnormal glucose, lipid, and bone metabolism, as well as atherosclerosis and neuronal degeneration, share similar molecular pathogenetic mechanisms implicated in the development of these conditions. This common regulation of metabolic homeostasis between different organs is largely due to the demonstration of a similar function of the same genes in different organs and organisms.\textsuperscript{[52]} Thus, it is possible that mechanisms of FoxO1 action have emerged to preserve and elicit the same metabolic effect through different organs, such as bone and pancreas. It is possible that under conditions where both the skeleton and glucose handling are deteriorating, such as in aging, FoxO1 may confer a rescuing signal of energy supply from the wasting skeleton to the energy-demanding organs that control glucose metabolism.

How could one integrate the antioxidant action with the metabolic action of FoxO1 on osteoblasts, and what would be the role of these two functions in physiology? FoxO1 activity in osteoblasts is under the opposing control of oxidative stress and insulin. The first stimulates whereas the second suppresses FoxO1 activity. It is possible that this dual mode of regulation may serve also as a dual rescue mechanism. In one mode, it preserves metabolic balance in conditions of increased oxidative stress. In the early stages of aging, a modest increase in oxidative stress can increase FoxO1 activity in bone, thus maintaining osteoblast numbers and preserving their function as endocrine cells that favor glucose availability. In another, opposite context,
in situations of metabolic stress (e.g., starvation) reduced insulin levels would lead to an increase in FoxO1 activity in bone. In turn, this increase in FoxO1 activity in bone would raise blood glucose levels, providing a source of nutrients to the brain. Thus, along with the pancreas and liver, bone becomes another organ that determines energy supply under stress.

Disclosures

The author states that she has no conflicts of interest.

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