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Review

FoxO1, the transcriptional chief of staff of energy metabolism

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ABSTRACT

FoxO1, one of the four FoxO isoforms of Forkhead transcription factors, is highly expressed in insulin-responsive tissues, including pancreas, liver, skeletal muscle and adipose tissue, as well as in the skeleton. In all these tissues FoxO1 orchestrates the transcriptional cascades regulating glucose metabolism. Indeed, FoxO1 is a major target of insulin which inhibits its transcriptional activity via nuclear exclusion. In the pancreas, FoxO1 regulates β-cell formation and function by a balanced dual mode of action that suppresses β-cell proliferation but promotes survival. Hepatic glucose production is promoted and lipid metabolism is regulated by FoxO1 such that under insulin resistance they lead to hyperglycemia and dyslipidemia, two features of type 2 diabetes. In skeletal muscle FoxO1 maintains energy homeostasis during fasting and provides energy supply through breakdown of carbohydrates, a process that leads to atrophy and underlies glycemic control in insulin resistance. In a dual function, FoxO1 regulates energy and nutrient homeostasis through energy storage in white adipose tissue, but promotes energy expenditure in brown adipose tissue. In its most recently discovered novel role, FoxO1 acts as a transcriptional link between the skeleton and pancreas as well as other insulin target tissues to regulate energy homeostasis. Through its expression in osteoblasts it controls glucose metabolism, insulin sensitivity and energy expenditure. In a feedback mode of regulation, FoxO1 is also a target of insulin signaling in osteoblasts. Insulin suppresses activity of osteoblastic FoxO1 thus promoting beneficial effects of osteoblasts on glucose metabolism. The multiple actions of FoxO1 in all glucose-regulating organs, along with clinical studies suggesting that its glycemic properties are conserved in humans, establish this transcription factor as a master regulator of energy metabolism across species.

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Introduction

Glucose homeostasis is a finely tuned process comprised of glucose production and uptake by several organs, with the pancreas and liver being the main stimulators or inhibitors of glucose production. Pancreatic β-cells rapidly sense elevations in blood glucose levels and respond by increasing insulin production through increased proliferation. The liver maintains blood glucose levels through two processes: gluconeogenesis, generation of glucose from non-carbohydrate carbon substrates, and glycogenolysis, degradation of glycogen. Skeletal muscle also regulates energy metabolism by contributing to more than 30% of resting metabolic rate and 80% of whole body glucose uptake. Adipocytes regulate energy homeostasis either through secretion of cytokines controlling appetite and insulin sensitivity or by storing

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excess amount of energy intake as triglycerides and mobilizing them to be oxidized during energy deprivation.

Until recently it was well accepted that glucose levels and energy expenditure are tightly coregulated by the classic peripheral insulin-producing or insulin-sensing organs. However, during the last few years, another organ has emerged as a potent regulator of energy metabolism, the skeleton [1–3]. In addition to its homeostatic properties during bone growth, the skeleton serves a novel endocrine function as a regulator of whole body glucose metabolism. In this task the skeleton regulates energy metabolism by favoring β-cell proliferation, insulin secretion, insulin sensitivity and energy expenditure. In addition, it is itself a target of insulin signaling [2,4]. Osteoblasts possess an intricate array of functions that control glucose metabolism through the secretion of the osteoblast-specific Osteocalcin, the activity of which is regulated in a bimodal mode of action. At the transcriptional level through suppression of the expression of *Esp*, a gene encoding a tyrosine phosphatase, which suppresses osteocalcin metabolic activity by promoting its carboxylation [5,6]. In addition insulin receptor (InsR) signaling in osteoblasts induces *Osteocalcin* expression by relieving the inhibitory effect of the transcription factor Twist2 on Runx2, a main regulator of *Osteocalcin* expression [2]. At the activity level, through direct regulation of osteocalcin carboxylation by another energy consuming function of the skeleton: bone resorption [4]. In a leap forward from the studies in rodents, many clinical studies have suggested that osteocalcin is a marker of glucose tolerance [1,2,7–10].

Demonstrating the pivotal role of bone in the control of energy homeostasis, recent evidence suggests that other hormones, in addition to osteocalcin may be mediating this endocrine function [3]. Indeed, partial osteoblast ablation in mice compromises glucose homeostasis and demonstrates that the skeleton potently regulates all determinants of energy metabolism: glucose and insulin production, glucose tolerance and insulin sensitivity, fat metabolism, energy expenditure and appetite in both osteocalcin-dependent and independent manners. The notion of an additional, bone-derived hormone regulating glucose metabolism is in line with the fact that other organs utilize more than one secreted molecules to affect body functions Remarkably for the skeleton, the exact same transcriptional mediator of insulin actions in all insulin-sensitive target organs also regulates the metabolic activity of osteocalcin and its insulin-upregulating as well as insulin-sensitizing actions: FoxO1 [6]. Thus, FoxO1 becomes a common unifying link of insulin signaling among all glucose-regulating organs (Fig. 1).

It is the dominant role of insulin signaling in all glucose-regulating organs that originally brought to light the Forkhead box O (FoxO) family of transcription factors. Among all transcription factors involved in energy regulation, the FoxO proteins, and more in particular FoxO1, are the main transcriptional modulators of insulin actions. Insulin suppresses FoxO1 activity through activation of the PI3K/AKT signaling pathway. Activated AKT phosphorylates FoxO1 at 3 highly conserved phosphorylation sites resulting in its nuclear exclusion and thus inhibition of transcription [11]. There are three additional FoxO proteins

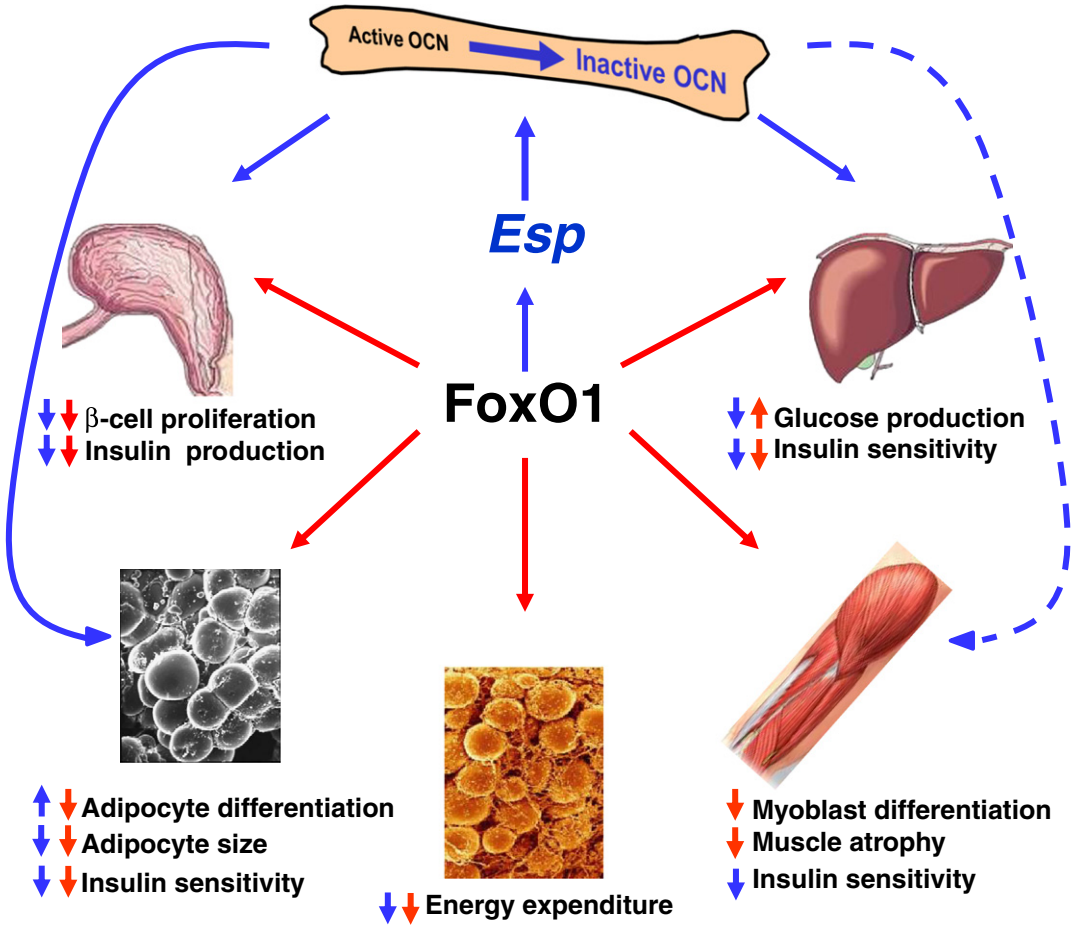

Fig. 1. FoxO1 is a unifying regulator of energy metabolism through the skeleton and peripheral organs. FoxO1 promotes glucose production by suppressing β-cell proliferation and insulin synthesis, by suppressing insulin sensitivity in the liver and white adipose tissue and by inducing expression of gluconeogenic enzymes. In the muscle, FoxO1 inhibits myoblastic differentiation but provides energy, when supplies are low, through breaking down of muscle protein leading to muscle atrophy. In white and brown adipose tissue FoxO1 has a dual function: in the first it decreases insulin sensitivity and suppresses adipogenesis and adipocyte size thus regulating energy and controlling energy storage. In the latter it decreases energy expenditure. In bone FoxO1 acts on osteoblasts to suppress expression of *Esp* and promote carboxylation/inactivation of Osteocalcin (OCN). Glucose levels increase through suppression of insulin production, decreased insulin sensitivity in the liver, muscle and white adipose tissue and suppression of energy expenditure. Although it decreases mitochondrial activity in the muscle, it is presently unknown whether if affects lean mass.

in mammals: FoxO3, FoxO4, and FoxO6. Both FoxO3 and FoxO4 contain the same conserved Akt phosphorylation sites as FoxO1. FoxO6 is regulated differently and is expressed primarily in brain [12]. FoxOs share a target consensus sequence and some overlapping functions [13], although some functions appear to be unique [14]. Notably, FoxO3- and FoxO4-null mice are viable, but FoxO1-null mice die in embryogenesis due to defects in arterial and venous development [15].

Among all the FoxO isoforms, FoxO1 is abundantly expressed in the pancreas, liver, skeletal muscle, white and brown adipose tissue and in the hypothalamus, all of the classic tissues that affect whole body energy homeostasis. FoxO1 is also the most highly expressed FoxO isoform in the skeleton that has, during the last few years, been identified as a novel regulator of energy metabolism and a target of insulin signaling [1,2,4]. Whether it is the fasting or fed state or in conditions of insulin resistance FoxO1 is activated and functions as a metabolic switch that shifts metabolic responses with the purpose of re-establishing energy homeostasis. During fasting FoxO1 promotes adaptation by inducing gluconeogenesis in the liver and a transition from carbohydrate oxidation to lipid oxidation in the muscle [16]. In the fed state hepatic and pancreatic FoxO1 is inhibited by insulin. This function in the liver shifts glucose metabolism to acetate for oxidation or conversion to fatty acids [17]. In the pancreas, FoxO1 inactivation is required for β-cell proliferation. In insulin resistance, FoxO1 activity is unleashed and thus inhibits the increase in β-cell proliferation that is needed to compensate the rise in insulin demand [18–20]. At the same time however, FoxO1 offers some protection by means of protecting β-cell function from increases in oxidative stress levels that parallel insulin resistance [21]. In bone FoxO1 exerts its glucose homeostatic functions by suppressing the activity of osteocalcin and thus suppressing insulin production and insulin sensitivity [6]. At the molecular level, FoxO1 fulfills these functions in all different tissues as a transcriptional modulator of insulin sensing genes as well as genes that are involved in lipid oxidation and metabolism, mitochondrial activity and energy uptake. It also controls the activity or production of hormones, either adipokines or an osteoblast-specific secreted protein, that regulate energy metabolism. The mechanisms by which FoxO1 affects glucose metabolism in peripheral organs and the skeleton are reviewed.

FoxO1 in the pancreas

Blood glucose levels are maintained within a narrow range under physiological conditions, partly by the action of hormones to stimulate or inhibit glucose production, which occurs mainly in the pancreas and liver [22]. Elevation of blood glucose levels or other nutrients are rapidly sensed by pancreatic β-cells, the archetypal metabolic sensors, which respond by secreting insulin or by increasing insulin production through increased proliferation. Insulin in turn activates a PI3K/AKT signaling cascade that acts mainly by downregulating FoxO1 activity to adapt to alterations in metabolic demands and to maintain nutrient homeostasis in the pancreas and the other peripheral tissues. FoxO1 regulates β-cell function in a dual mode of action. It suppresses β-cell mass either by suppressing proliferation during hyperglycemia or insulin resistance, or by suppressing differentiation in the developing fetal pancreas. On the other hand, FoxO1 protects β-cells from oxidative stress-induced damage due to glucose or lipid loading.

Among the many factors that regulate β-cell development and mass, pancreatic and duodenal homeobox factor-1 (Pdx1) is present in all pancreatic cells during embryonic development and is restricted to β-cells in the adult pancreas [23]. It regulates the expression of several β-cell genes including the β-cell glucose transporter Glut2 and glucokinase. FoxO1 negatively regulates *Pdx1* expression by competing with the transcription factor FoxA2 for binding to the *Pdx1* promoter [18]. This effect along with downregulation of *neurogenin 3*, required for the development of the four endocrine cell lineages of the pancreas, and *Nkx61*, required for β-cell development, mediate the suppressive effect of FoxO1 on β-cell differentiation [24]. At the same time nuclear translocation of FoxO1 tends to exclude Pdx1 from the nucleus and this serves as an alternative mechanism to suppress β-cell proliferation [18]. However, knocking out *FoxO1* at different times during β-cell development leads to vastly different phenotypes, suggesting that FoxO1 may have different functions at different stages of pancreas development [25]. In addition to suppressing expression of transcription factors regulating pancreatic development, FoxO1 is a negative regulator of insulin sensitivity in β-cells. Insulin, by activating the PI3K/AKT signaling cascade, induces FoxO1 phosphorylation at 3 highly conserved AKT phosphorylation sites. This results in inhibition of FoxO1 translocation to the nucleus or FoxO1 sequestration from the nucleus to the cytosol and thus inhibition of transcription.

Loss-of-function animal models of FoxO1 have clearly demonstrated that it suppresses β-cell proliferation and function [19,26]. Removal of one *FoxO1* allele from mice with β-cell-specific inactivation of *Pdx1*, rescues the defects in β-cell development [27]. Additionally, FoxO1 haploinsufficiency partially reinstates the decreased β-cell proliferation observed in Insulin receptor substrate-2 (*Irs2*) knockout mice [18] as well as in mice with β-cell-specific deletion of *Pdk1*, a PI3K-dependent protein kinase that is important for maintenance of β-cell mass [27]. That FoxO1 is a negative regulator of insulin signaling in β-cells *in vivo*, is supported by the fact that selective overexpression of AKT in β-cells increases β-cell survival and size [28]. Recently, overexpression of FoxO1 in pancreatic cells cultured under low nutrition conditions promoted their proliferation through induction of cyclin D1 expression [29].

Failure of β-cell function is commonly seen in type 2 diabetes. Although the nature of the underlying metabolic abnormalities is not yet clear, and probably multifactorial, a widely held theory is that chronic exposure to elevated glucose levels causes deterioration of β-cell function, a phenomenon known as “glucose toxicity” [30]. Glucose toxicity is thought to arise as a consequence of chronic oxidative stress, when intracellular glucose concentrations exceed the glycolytic capacity of the β-cell. Under these conditions, glucose is shunted to the enolization pathway, resulting in the generation of superoxide anions and induction of apoptosis [31]. In contrast to its suppressive effects on β-cell proliferation, FoxO1 preserves β-cell function under oxidative stress-induced damage [26]. Oxidative insult overcomes the inhibitory effect of insulin/PI3K/AKT signaling on FoxO1 nuclear translocation by two potential mechanisms that involve activation of JNK. Activated JNK can either directly inhibit insulin-induced AKT activation or promotes translocation of FoxO1 to the nucleus of pancreatic β-cells [32]. Nuclear FoxO1 is targeted to promyelotic leukemia protein (Pml)-containing subdomains where it is deacetylated by the NAD+-dependent deacetylase Sirt1 and increases expression of the *Ins2* gene transcription factors NeuroD and MafA [26].

In conclusion, in physiological conditions FoxO1 regulates β-cell formation and function by a dual mode of action that requires a balanced FoxO1 activity. Activated FoxO1 suppresses β-cell proliferation but promotes survival by increasing stress resistance. Thus, either hyperactivated or hypoactivated FoxO1 could result in β-cell failure.

FoxO1 in hepatic glucose production

Hepatic glucose production (HGP) is a combination of gluconeogenesis and glycogenolysis, and although it is generally accepted that HGP is high in patients with diabetes, the physiology of this abnormality remains disputed. Insulin inhibits glucose production by indirect and direct mechanisms. The latter result in transcriptional suppression of the key gluconeogenetic and glycogenolytic enzymes, phosphoenolpyruvate carboxykinase (*Pepck*) and glucose-6-phosphatase (*G6pc*). FoxO1 confers hormone sensitivity and promotes HGP [33]. In hepatocytes, FoxO1 interacts with the Pparγ coactivator Pgc1α to promote the expression of *Pepck* and *G6pc* [34]. This activity is blocked

by insulin through the PI 3-kinase (PI3K)-Akt pathway, wherein phosphorylation of FoxO1 by Akt causes its nuclear exclusion and inactivation [35]. Accordingly, FoxO1 haploinsufficiency or hepatic expression of dominant negative FoxO1 in mice reduces gluconeogenic gene expression and rescues the diabetic phenotype of insulin resistance [19,34]. Mice with liver-specific inactivation of FoxO1 show reduced gluconeogenic gene expression, improved glucose tolerance, and reduced HGP in hyperinsulinemic clamps [36]. Moreover, hepatic FoxO1 ablation in mice lacking either the insulin receptor or the insulin receptor substrates IRS1 and IRS2 rescues the diabetic phenotypes and hepatosteatosis of these mice [17,36]. FoxO1 is also part of a transcriptional interplay that regulates gluconeogenesis during prolonged periods of fasting. Glucagon stimulates the gluconeogenic program by activating the CREB regulated transcription coactivator 2 (CRTC2), while parallel decreases in insulin signaling augment gluconeogenic gene expression through activation of FoxO1. During prolonged fasting the Sirt1 histone deacetylase deacetylates CRTC2 promoting its degradation but upregulates FoxO1 activity. This reciprocal regulation of transcription by FoxO1 and CRTC2 by Sirt1 seems to be critical in maintaining energy balance during fasting [37].

Ablation of all three FoxOs in liver causes considerably greater defects in glucose metabolism, beyond those in the single FoxO1 liver knockout mice [38]. Interestingly, compound *FoxO* knockout mice did not display further decreases in FoxO1 targets. Instead, they showed that some targets, such as *G6pc* and *Igfbp1*, may be preferentially regulated by FoxO1, whereas other targets, such as glucokinase (*Gck*), that contribute to glucose homeostasis are regulated redundantly by multiple FoxOs. This suggests that the further reduction in glucose output in these mice as compared to the FoxO1 knockouts may be due to a combination of reduced gluconeogenesis and rapid metabolism of glucose into glucose-6-phosphate.

Alterations in lipid metabolism are also an integral part of diabetes and the metabolic syndrome that can lead to development of cardiovascular diseases [39]. The primary abnormalities in lipid metabolism are increased plasma triglycerides and cholesterol in atherogenic lipoprotein fractions, in part due to excessive secretion of VLDL by the liver, and increased free fatty acid (FFA) levels [40]. In addition to its effects in gluconeogenesis, FoxO1 also mediates insulin action on triglyceride metabolism. Indeed, mice expressing the constitutively active form of FoxO1 showed increased expression of liver and apolipoprotein C-III (*apoC-III*) and increased production of liver and plasma triglycerides [41]. In contrast, antisense oligonucleotide-mediated inhibition of FoxO showed reduced levels of liver triglycerides. FoxO1 also controls aspects of lipid metabolism in the diabetic liver. Whereas insulin resistance fails to inhibit FoxO1-driven gluconeogenesis it promotes lipogenesis (dyslipidemia). Hyperactivated FoxO1 in this setting of insulin resistance contributes to the accumulation of hepatic lipids. It promotes hepatic triglyceride accumulation that is associated with decreased fatty acid oxidation and can progress to steatosis [42]. FoxO1 gain of function in transgenic mice or loss-of-function in diabetic, *db*/*db* mice indicate that FoxO1 through enhancing the expression of hepatic microsomal triglyceride transfer protein (MTP), regulates the rate limiting step of assembly of triglyceride very low density lipoproteins (VLDL) [43]. Hepatic FoxO1 ablation in diabetic mice increases cholesterol, plasma FFA and VLDL secretion, the three hallmarks of the diabetic state [44]. These effects correlate with increases in the expression of liver *Fgf21*. Because increased liver FGF21 stimulates release of FFAs from adipose tissue [45], FoxO1 may be limiting FFA release from adipose tissue. Based on additional changes in gene expression, it was proposed that FoxO1 by controlling lipolysis and the availability of substrates for triglyceride and cholesterol synthesis and VLDL secretion, protects against excessive hepatic lipid production during hyperglycemia. Thus, paradoxically, inhibition of FoxO1 activity by insulin treatment may exacerbate the lipid abnormalities of diabetes.

In summary, activated FoxO1 promotes HGP through induction of the gluconeogenic enzymes *Pepck* and *G6Pase*. It also participates in lipid metabolism. Under insulin resistance hyperactive FoxO1 causes hyperglycemia and dyslipidemia, the characteristics of diabetes and diabetic complications. Therefore, finely tuned FoxO1 activity is a premise of nutrient and metabolic homeostasis.

FoxO1 regulates skeletal muscle atrophy and glycemic properties

Skeletal muscle also regulates energy metabolism by contributing to more that 30% of resting metabolic rate and 80% of whole body glucose uptake [46]. Skeletal muscle is formed by a succession of steps that start by commitment to the myoblast lineage, an event requiring activation of myogenic transcription factors and subsequent myoblast fusion to form mononucleated myoblasts. Myoblast differentiation initiates with the expression of *MyoD* the earliest marker of muscle cell differentiation as well as several other muscle specific proteins such as *myosin* and *myocyte enhancer factor-2* (*MEF-2*). Finally, mononucleated myoblasts fuse to form muscle fibers, which according to the particular subtype of myosin heavy chain contained are classified in slow- (type I) or fast- (type II) twitch myofibers [47].

FoxO1 is involved in the proliferation of myoblasts, the fusion of mononucleated monocytes into myotubes, in myogenic lineage specification and in the breakdown of muscle fibers. In proliferating myoblasts FoxO1 remains inactive, presumably through a PI3K/AKT-independent mechanism of nuclear exclusion. Instead, FoxO1 inactivation is potentially due to direct phosphorylation by the Rho-associated kinase ROCK a downsteam effector of the small GTPase Rho which is required to maintain extranuclear localization of FoxO1 and at the same time suppresses myoblast differentiation [48]. However, once in the nucleus FoxO1 promotes myotube formation by upregulating the expression of the genes involved in cell fusion [49].

Mice overexpressing FoxO1 show downregulation of slow-twitch muscle genes suggesting that FoxO1 determines myogenic lineage specification [50]. Consistent with this key function in the myogenic program, FoxO1 ablation in the muscle results in a fiber type switch to MyoD-containing fast-twitch myofibers and altered fiber type distribution at the expense of myogenin-containing, slow-twitch myofibers [51]. At the same time FoxO1 suppresses MyoD-dependent myogenesis in cultures of myoblasts. These effects are mediated by a functional and physical interaction of FoxO1 with Notch1 which leads to corepressor clearance from the Notch effector Csl, leading to activation of Notch target genes. This role of FoxO1 is independent of its transcriptional function and involves a direct interaction with Csl and subsequent stabilization of a FoxO1/Notch1 complex.

In vivo studies of FoxO1 inactivation or overexpression have showed that it affects to a great extend skeletal muscle mass. Mice overexpressing FoxO1 lose their glycemic control due to a decrease in skeletal muscle mass [50]. In addition to suppression of the myogenic program, this effect is associated with systemic muscle atrophy, a condition that results from breakdown of muscle fibers. Indeed, transgenic overexpression of FoxO1 in skeletal muscle results in severe muscular atrophy [52]. These effects of FoxO1 are mediated by its actions in three genes, *atrogin-1* and *myostatin* and *4E-binding protein-1*. FoxO1 promotes the expression of *atrogin-1*, a muscle-specific ubiquitin ligase which along with MuRF1 controls muscle atrophy [53]. Indeed, inactivation of FoxO1 in myotubes or rodent muscle decreases both muscle atrophy and *atrogin-1* expression. In addition, constitutive activation of FoxO1 in the same cells enhances the transcriptional activity of myostatin, another muscle-specific protein causing muscle loss, through direct binding of FoxO1 to the myostatin promoter [54]. Finally, FoxO1 induces the translational inhibitor 4E-binding protein-1 by binding to its promoter, thereby suppressing protein synthesis [52]. Expression or activity of FoxO1 is not affected in the muscle of patients with amyotrophic lateral sclerosis, a condition of chronic muscular atrophy, in spite of increased *atrogin-1* levels and decreased AKT

phosphorylation [55]. However, they are affected during exercise and de-training, and during starvation, conditions that alter skeletal muscle [56], thus suggesting that conditions that promote muscle atrophy also promote FoxO1 expression or activity.

Skeletal muscle metabolism appears to also be affected by FoxO1, through its action on the expression of three enzymes which during fasting regulate the switch from oxidation of carbohydrates as a major energy source to fatty acid. In states of energy deprivation FoxO1 promotes the expression of pyruvate dehydrogenase kinase-4 (*Pdk4*), the enzyme that shuts down glucose oxidation by blocking the activity of pyruvate dehydrogenase [49]. At the same time, over-expression of FoxO1 in C2C12 myoblasts increases the expression of *lipoprotein lipase* which hydrolyzes plasma triglycerides into fatty acids; and, enhances the plasma levels of fatty acid translocase CD36 facilitates fatty acid uptake into skeletal muscle [16]. The latter two events promote fatty acid uptake and oxidation.

In general FoxO1 expression or activity is induced during fasting as a means of maintaining energy homeostasis through utilization of lipids rather than carbohydrates as the energy source in the muscle. In starvation, FoxO1 provides energy supply through breaking down of muscle protein, an action that leads to muscle loss and atrophy and underlies glycemic control in insulin resistance.

FoxO1 regulates energy storage and expenditure through adipose tissue

Adipose tissue has a central role in the regulation of energy homeostasis. Adipocytes serve as lipid reservoirs storing excess amount of energy intake as triglycerides and mobilizing them to be oxidized during energy deprivation [57]. In addition they secrete adipokines that control energy homeostasis through actions on them, the brain or other tissues. When energy intake exceeds energy expenditure obesity arises. In turn obesity leads to insulin resistance in peripheral tissues due to altered expression or secretion of adipokines and infiltration of macrophages and subsequently causes type 2 diabetes, hyperlipidemia and hypertension and finally atherosclerosis [58].

FoxO1 suppresses adipogenesis. Expression of a non-phosphorylatable constitutively active FoxO1 prevents the differentiation of a preadipocytic cell line [59]. Consistently, a dominant negative FoxO1, with a truncated C-terminal transactivation domain, restores adipocyte differentiation of embryonic fibroblasts from insulin receptor knockout mice and promotes adipogenesis *in vitro* [60]. FoxO1 haploinsufficiency restores adipocyte number and size in mice fed a high fat diet [61]. In transgenic mice, inhibition of FoxO1 activity in white adipose tissue improves glucose tolerance and insulin sensitivity and increases energy expenditure under normal as well as high fat diet [60]. Selective inhibition of FoxO1 in brown adipose tissue enhances oxygen consumption and the expression of genes promoting mitochondrial metabolism, *Pparγc1* and *UCP1*. The anti-adipogenic actions of FoxO1 appear to be under the control of insulin signaling as cells lacking either insulin receptor, or insulin receptor substrate or the insulin target AKT, have increased FoxO1 activity and impaired differentiation [62–64]. Acetylation status is another factor affecting FoxO1 activity. Sirt2 and Sirt1, two NAD-dependent deacetylases, prevent adipocyte differentiation and at the same time deacetylate and subsequently prevent FoxO1 nuclear exclusion, indicating that FoxO1 may be involved in these suppressive effects [65].

FoxO1 exerts its adipogenesis-suppressing actions at the early stages of adipocyte differentiation and at the end stage of clonal expansion and terminal differentiation by induction of cell cycle arrest, through upregulation of the cell cycle inhibitor p21 (89). In addition, FoxO1 also regulates the expression and activity of two master adipogenic transcription factors, PPARγ and C/EBPα. It suppresses both PPARγ expression, through direct binding to the promoter, and PPARγ activity by competitively inhibiting the formation of the PPARγ/RXR functional complex [66]. On the other hand it physically interacts with C/EBPα to promote expression of adiponectin [59].

These observations suggest that FoxO1 has a dual function in white and brown adipose tissue. Whereas in the first it regulates energy and nutrient homeostasis through energy storage, in the latter it regulates energy expenditure.

FoxO1 is a transcriptional modulator of the energy-regulating properties of the skeleton

During the last few years, the skeleton has been identified as a dynamic, interactive organ which receives and transmits regulatory signals from and to other tissues. These observations have expanded the homeostatic role of the skeleton beyond the regulation of bone growth and remodeling to a novel endocrine function as an important regulator of whole body glucose metabolism. In this function the skeleton regulates energy metabolism by favoring β-cell proliferation, insulin secretion, insulin sensitivity and energy expenditure [1,2,4]. In addition, it is a target of insulin signaling. Osteoblasts are the cells orchestrating these responses through the secretion of osteocalcin. Osteocalcin, in its under- or un-carboxylated form, acts as a hormone to promote hyperinsulinemia and improve insulin sensitivity, a combination that results in improved glucose tolerance and glucose metabolism. Several clinical studies have now come forward supporting the notion that osteocalcin is a marker of glucose tolerance [1,2,7–10]. In a tight regulatory mechanism, not only the skeleton signals to the pancreas and affects insulin signaling in other insulin-target tissues but insulin signaling is also operating in osteoblasts to regulate bone remodeling [4]. Insulin acts on osteoblasts and through them to promote osteoclast activity and bone resorption. The acidity of the resorptive environment decarboxylates osteocalcin to unleash its stimulatory effect on insulin production and glucose metabolism.

Bearing in mind the role of FoxO1 as a main target of insulin signaling in all insulin-sensitive target organs and the novel endocrine function of the skeleton, it was unavoidable to ask the following questions: could FoxO1 fulfill its metabolic functions in part through its expression in osteoblasts? And if that were true, could this be due to its ability to act as a transcriptional modulator of osteocalcin. A mouse model with osteoblast-specific inactivation of FoxO1 shed light to the first question by showing that FoxO1 acts on osteoblasts to suppress pancreatic islet proliferation and function and insulin sensitivity in its peripheral target organs and as a result to raise blood glucose levels in both the fed and fasting states [6]. In addition, and consistent with its hypoinsulinemic actions, osteoblast-expressed FoxO1 promotes gluconeogenesis without affecting any counter-regulatory hormones with anti-insulinemic activity such as glucagon or growth hormone.

Mice lacking *FoxO1* in osteoblasts had an improved glucose disposal load and glucose tolerance which was also related to the favorable glucose metabolism by the liver, muscle and white adipose tissue [6]. This was suggested by the fact that expressions of several insulin target genes that also regulate lipid oxidation were increased in these tissues. In the liver expression of *FoxA2*, which regulates lipogenesis and ketogenesis during fasting, was increased whereas expression of *G6Pase* and *Pepck1* was decreased in mice lacking FoxO1 in osteoblasts. Muscle mitochondrial activity was also increased as indicated by increased expression of *Pgc1α* and its target genes, *Nrf1* and *Mcad* which are associated with mitochondrial activity. Indeed, FoxO1 osteoblast deletion leads to a higher ATP: ADP ratio in the muscle due to an increase in ATP production and a concomitant decrease in AMP. In addition, expression of biomarkers in the oxidative phosphorylation metabolic pathway that uses energy released by the oxidation of nutrients to produce ATP [67] was also altered. Expression of *uncoupling protein 3* (*Ucp3*), muscle *carnitine palmitoyl transferase I* (*mCPTI*) and *pyruvate dehydrogenase kinase 4* (*Pdk4*), the mitochondrial respiratory chain proteins mtDNA-encoded subunits: subunit 6 of NADH dehydrogenase (ND6, complex I) and subunit I of cytochrome c oxidase (COXI,

complex IV) were all upregulated indicating stimulation of mitochondrial activity. Gonadal fat pad weight was increased, in spite of the hyperinsulinemia and insulin sensitivity, related, at least in part, to the fact that energy expenditure and activity levels were increased whereas energy intake was not affected. Expression of the adipogenic gene *C/EBPα* and two lipolytic genes *Perillipin* and *Triglyceride lipase* was decreased suggesting that adipogenesis and lipolysis may be decreased with FoxO1 deletion in osteoblasts. Indicating a therapeutic relevance of these findings, hyperinsulinemia and increased insulin sensitivity in mice lacking *FoxO1* in osteoblasts could be effective in protecting these animals from obesity, and obesity-induced glucose intolerance and insulin resistance.

At the molecular level, at least part of the metabolic actions of FoxO1, are due to its ability to promote osteocalcin expression but mainly carboxylation and thus suppress osteocalcin activity [6]. Indeed, osteocalcin activity, measured by the percentage of its uncarboxylated form in the serum, and *osteocalcin* expression were increased in mice lacking *FoxO1* in osteoblasts. FoxO1 regulates *osteocalcin* expression through direct binding to FoxO1 binding sites present in the promoter as well as the 1st intron of the *osteocalcin* gene. Regulation of osteocalcin activity is indirect through down-regulation of the expression of *Esp*, the gene encoding OST-PTP which influences osteocalcin function by promoting γ-carboxylation. The stimulatory effect of FoxO1 on *Esp* expression results, at least in part, from direct binding of FoxO1 to the 1st intron of *Esp*. More importantly, a series of genetic experiments using compound mutant mice showed that removal of one allele of *osteocalcin* from mice lacking *FoxO1* from osteoblasts, corrects the metabolic phenotype of improved glucose tolerance and insulin sensitivity that results from osteoblast-specific inactivation of FoxO1. Thus, FoxO1 controls glucose metabolism through the osteoblasts, at least in part, by regulating the activity of osteocalcin. In addition to FoxO1, another transcription factor, ATF4, is also a regulator of glucose metabolism through its expression in osteoblasts [5]. Genetic studies have showed that the two transcription factors synergize in osteoblasts to confer their glucose regulating properties (our unpublished observations).

Finally, not only osteoblast-expressed FoxO1 regulates insulin production and sensitivity, but in a feedback mode of regulation, FoxO1 is also a target of insulin signaling in osteoblasts. Insulin receptor signaling in osteoblasts inactivates FoxO1 in a PI3K/AKT-dependent manner. FoxO1 inactivation favors Osteocalcin activity in a dual mode of action. On one hand, it downregulates expression of *Esp* thereby promoting Osteocalcin decarboxylation [6]. On the other hand, it reduces production of the anti-osteoclastogenic factor Osteoprotegerin, and promotes osteoclastogenesis and bone resorption [4]. It appears that through these feedback pathways, one at the transcriptional level with FoxO1 and the other at the hormonal level with osteocalcin, the skeleton and pancreas interact to tightly, and perhaps redundantly in cases of need, regulate energy metabolism.

Perspective: A translation to metabolic disease in humans

It is clear from the studies in cells and the various genetic models that FoxO1 affects metabolism at multiple levels and in many mechanisms. Recent evidence suggests that these observations relate to metabolic regulation in humans. In clinical studies, analysis of the genetic variance in *FOXO1a* and *FOXO3a* on metabolic profiles, age-related diseases and mortality have indicated higher HbA1c levels and increased mortality risk associated with specific haplotypes of *FOXO1a* as well as increased glucose levels and a trend for early onset type-2 diabetes [68–70]. These observations may be relevant to the demonstration that elevated glucose levels can suppress phosphorylation of FOXO1, FOXO3a (and FOXO4) in human endothelial progenitor cells and promote their apoptosis [71]. More specifically with regards to metabolic dysregulations, whereas a *FOXO1a* haplotype has been associated with lower risk to develop type 2 diabetes [72], a common genetic variation within *FOXO1a* has been associated with alterations in insulin secretion and glucose tolerance and an increased risk of type 2 diabetes [73]. The two latter opposing observations may simple indicate gain- or loss- of function mutations in *FOXO1a*. Alternatively, it may simply indicate the various roles of FoxOs in different tissues. FoxO proteins may also protect against diminished mitochondrial energy levels known to occur during insulin resistance such as in the elderly populations [74]. This clinical evidence suggests that the human homologue of FoxO1 has the same impact in humans and as such may influence glucose metabolism and contribute to predisposition to diabetes. Thus, identifying specific FoxO1 targets that mediate the metabolic functions of FoxO1 in the skeleton and peripheral tissues will not only help to better understand mechanisms of compromised glucose metabolism but may also reveal novel pharmacological targets. Indeed in the case of the skeleton this prediction seems to be on its way to validation. A growing number of studies show that serum osteocalcin is inversely correlated with fat mass and plasma glucose levels in adult and elderly individuals [7,8,10]; and, positively correlated with beta cell function in diabetic patients, before and after glycaemic control [75,76]. In addition, obese individuals have lower osteocalcin levels as compared to non-obese ones, and type 2 diabetic individuals have lower plasma osteocalcin [8,77]. This may be related to another inverse correlation of serum osteocalcin with the adipogenic adipocyte-specific fatty acid-binding protein (A-FABP), and with HDL-cholesterol in men, and a positive correlation with adiponectin levels in post-menopausal women [9,75].

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