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An overview of the metabolic functions of osteocalcin

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Abstract

A recent unexpected development of bone biology is that bone is an endocrine organ contributing to the regulation of a number of physiological processes. One of the functions regulated by bone through osteocalcin, an osteoblast specific hormone, is glucose homeostasis. In this overview, we explain the rationale why we hypothesized that there should be a coordinated endocrine regulation between bone mass and energy metabolism. We then review the experiments that identified the endocrine function of osteocalcin and the cell biology events that allow osteocalcin to become a hormone. We also demonstrate the importance of this regulation to understand whole-body glucose homeostasis in the physiological state and in pathological conditions. Lastly we discuss the epidemiological and genetic evidence demonstrating that this function of osteocalcin is conserved in humans.

Keywords

Osteoblast; Energy metabolism; Osteocalcin

1 Introduction

The hypothesis that bone is an endocrine organ regulating, among other functions, energy metabolism arises from a conceptual view of bone biology anchored in evolution. Importantly, this view of bone biology is supported by both clinical observations and experimental evidence.

We interpreted the fact that bone is the only tissue that contains a cell type, the osteoclast, whose only function is to destroy or to resorb mineralized bone matrix as being of fundamental importance. By definition, because it occurs daily in a tissue that covers a large surface in our body, this active destruction of mineralized bone requires energy [1, 2]. Presumably the quantity of energy this active destruction of mineralized bone requires is proportional to the surface occupied by bone. This energetic requirement is probably very high since bone resorption does not occur in isolation but in the context of a bi-phasic physiological function called bone modeling during childhood and bone remodeling during adulthood [3]. In this function, bone resorption is followed by bone formation, a cellular

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process that relies on the daily synthesis of proteins; hence, bone formation also requires energy [4]. This is why we have hypothesized that bone modeling and remodeling have to be linked to the regulation of energy metabolism. This view of bone biology that infers a coordinated regulation of bone mass accrual and energy metabolism is supported by clinical observations. For instance, when food, i.e. energy intake is severely decreased during childhood there is an arrest of growth; when this situation develops in adults there is bone loss [5–15].

2 An unexpected experimental observation

Even if tantalizing, the hypothesis that there may be a coordinated regulation of bone mass accrual and energy metabolism would probably not have been tested any further if it was not for a rather unexpected experimental evidence. *Osteocalcin* is an osteoblast-specific gene encoding a secreted protein that was identified in the late 70's but whose functions in the pre-model organism era of biology were unknown [16–18]. Hypothesizing, probably naively and as it turned out wrongly, that it may regulate bone mineralization, we generated 20 years ago *Osteocalcin*-null mice [19, 20]. The first result we obtained was certainly disappointing to us since bone mineralization is essentially normal in the absence of *Osteocalcin* [20]. That is not to say however that osteocalcin has no functions or that the *Osteocalcin*^{−/−} mice had no phenotype. Indeed, every time we sacrificed *Osteocalcin*^{−/−} mice we made the same observation: they had visibly more visceral fat than wild type littermates. In addition *Osteocalcin*^{−/−} mice were poor breeders. Since osteocalcin is only made in osteoblasts these observations inferred from the onset that bone might be an endocrine organ and that one hormone it secretes, osteocalcin, somehow affects fat mass and fertility. It is these experimental observation that when confronted to the conceptual view of bone (re)modeling and the clinical observations presented above, led to the hypothesis that there is a coordinated regulation, endocrine in nature, of bone mass, energy metabolism and reproduction. An inference of fundamental importance of this hypothesis is that bone should be an endocrine organ and not just a recipient of hormonal intake. This latter tenet of the hypothesis was consistent with the phenotypic abnormalities seen in *Osteocalcin*^{−/−} mice.

3 Identification of osteocalcin as a hormone regulating insulin secretion

An additional and serendipitous observation that we made 10 years later further suggested that the osteoblast is an endocrine cell type regulating energy metabolism and more specifically glucose metabolism. We had generated mice lacking a tyrosine phosphatase expressed only in osteoblasts and Sertoli cells of the testis, hence its name, osteoblast testis specific protein tyrosine phosphatase (OST-PTP) [21]. This receptor protein tyrosine phosphatase is encoded by a gene termed *Esp* [22]. Importantly, whether the gene was deleted in all cells or in osteoblasts only, *Esp*^{−/−} mice exhibited the same phenotype made of hypoglycemia, hyperinsulinemia and increased glucose utilization by peripheral tissues [23]. Moreover, mice lacking *Esp* in all cells or in osteoblasts only had much less visceral fat. These findings established in an unambiguous manner that the osteoblast was an endocrine cell type regulating one particular aspect of energy metabolism: glucose homeostasis. However, since OST-PTP is not a secreted protein, these observations implied the existence of another molecule, presumably a hormone, made by osteoblasts and

regulating glucose homeostasis. The fact that *Esp*^{-/-} mice had a low fat mass phenotype i.e. a phenotype that was exactly the mirror image of what was observed in *Osteocalcin*^{-/-} mice, led us to test whether OST-PTP could inhibit osteocalcin function. This revived, 10 years later, our interest in the hypothetical endocrine function of osteocalcin but this time with a more defined and testable hypothesis.

The demonstration that osteoblasts are endocrine cells stimulating insulin secretion and that this function was fulfilled by osteocalcin came from a classical cell biology experiment [23]. Indeed, a co-culture of mouse osteoblasts and mouse pancreatic islets resulted in an increase in *Insulin* expression in islets. Several controls indicated this was a meaningful result. For instance when this co-culture experiment was performed using a filter allowing transfer of small molecules but preventing cell-cell contact, the increase of *Insulin* expression in islets co-cultured with osteoblasts was still observed. In contrast, this insulin secretion ability was specific of osteoblasts since the closest relative to an osteoblast, a fibroblast, could not enhance *Insulin* expression in pancreatic islets. Third, osteoblasts did not increase the expression of any other hormones synthesized by pancreatic islets. Last but not least, when this experiment was repeated with *Osteocalcin*^{-/-} osteoblasts instead of WT ones, the favorable effect of osteoblasts on *Insulin* expression was virtually abolished although not completely. Conversely, when *Esp*^{-/-} osteoblasts were used in this assay, the increase in *Insulin* expression was significantly greater than when islets were co-cultured with WT osteoblasts. The notion that osteocalcin was an osteoblast-derived hormone regulating insulin secretion was further strengthened by showing that forced expression of *Osteocalcin* in COS cells conferred to these cells an ability to induce insulin secretion in a co-culture assay that they did not have otherwise.

Thus, this series of classical cell biology experiments demonstrated that osteoblasts are endocrine cells regulating *Insulin* expression, identified osteocalcin as an osteoblast-derived hormone responsible of this function, and revealed the existence of a genetic pathway taking place in osteoblasts and in which the receptor tyrosine phosphatase OST-PTP encoded by *Esp* inhibits, through mechanism that had to be uncovered, the ability of osteoblasts to favor *Insulin* expression in pancreatic islets. All these conclusions drawn from these cell biology assays were verified *in vivo* through genetic means.

Going back to an *in vivo* analysis, we then showed that *Osteocalcin*^{-/-} mice fed a normal chow were hyperglycemic and hypoinsulinemic [23]. A glucose stimulated insulin secretion test (GSIS) showed that insulin secretion was decreased in the absence of *Osteocalcin*, whereas it was increased in the absence of *Esp*. Consistent with this *in vivo* observation, β -cell mass, β -cell area and insulin content were decreased in *Osteocalcin*^{-/-} mice and increased in *Esp*^{-/-} mice. A glucose tolerance test (GTT) showed that *Osteocalcin*^{-/-} mice were glucose intolerance, in part, because of a decrease in *Insulin* expression. Again, *Esp*^{-/-} mice had exactly the opposite phenotype. Lastly and although it is not directly relevant to the topic of this review that focuses only on the bone-pancreas crosstalk, an insulin tolerance test (ITT) and euglycemic hyperinsulinemia clamp analysis showed that *Osteocalcin*^{-/-} mice were resistant to insulin signaling in several peripheral tissues while *Esp*^{-/-} mice were more sensitive to insulin signaling than WT mice.

The demonstration that *Esp* acts upstream of osteocalcin and inhibits its endocrine functions was provided by a genetic epistasis experiment. We reasoned that if the reason why *Esp*^{-/-} mice are able to secrete more insulin following a glucose challenge and are more tolerant to glucose than WT mice is because they have higher circulating osteocalcin levels, then removing one copy of *Osteocalcin* from these *Esp*^{-/-} mice should normalized osteocalcin circulating levels, insulin secretion and glucose tolerance. This prediction was verified by the analysis of *Esp*^{-/-};*Osteocalcin*^{+/-} mice which showed normal glucose homeostasis due to a decrease in circulating osteocalcin levels. In other words and even though they are a mouse model of loss of function of a particular gene, *Esp*^{-/-} mice are a model of gain-of osteocalcin function. This turned out to be quite important as it provided an internal control for all subsequent experiments addressing one function or another of osteocalcin. In each case we could rely on a loss-of function model, the *Osteocalcin*^{-/-} mice and a gain-of function model, the *Esp*^{-/-} mice that should have the opposite phenotype. In closing we must clearly reiterate that osteocalcin regulation of glucose metabolism is not synonymous of bone only origin of diabetes. It is simply a broadening of our understanding of the regulation of glucose metabolism and of bone biology as a whole.

4 A post-translational modification modulates osteocalcin bioactivity

As it is the case for any novel hormone, the demonstration that osteocalcin was a hormone regulating insulin secretion and glucose homeostasis among other functions raised several novel questions. Chief among them were: How does osteocalcin act to regulate insulin secretion and glucose tolerance in other words what is its receptor? Can we extend these mouse-based findings to human through epidemiological and/or genetic means? There were in addition to these questions, two other ones that were more specific to osteocalcin biology. Given that osteocalcin is subjected to a post-translational modification, a gamma carboxylation of some glutamate residues, what exactly is the mechanism whereby OST-PTP, the gene product of *Esp*, inhibits osteocalcin endocrine functions and which form of osteocalcin is fulfilling its endocrine functions? For obvious reasons that have to do with experimental simplicity, this latter question was the first one to be answered.

Osteocalcin is carboxylated on three glutamine acid residues within the osteoblasts before being released into the bone extracellular matrix, however, both the carboxylated and uncarboxylated forms of osteocalcin can be found in the general circulation [24]. Since the gamma carboxylase enzyme responsible of this post-translational modification is not expressed in bacteria, the use of recombinant, bacterially produced osteocalcin, allowed to address this aspect of osteocalcin biology. Recombinant and therefore uncarboxylated osteocalcin, but not carboxylated one, was able to induce *Insulin* expression in pancreatic islets thus indicating that it is the uncarboxylated form of osteocalcin that is acting as a hormone [23, 25]. Consistent with this notion, this form of osteocalcin is significantly more abundant in the serum of *Esp*^{-/-} mice than in the serum of WT mice [23, 26].

5 Identification of insulin signaling in osteoblasts as a mean to regulate osteocalcin bioactivity

How could *Esp* that encodes an intracellular enzyme, regulate the activity of a secreted molecule like osteocalcin? This was a more burning question considering that OST-PTP is a tyrosine phosphatase but neither osteocalcin nor the enzymes responsible of its carboxylation are phosphorylated [27]. This led us to consider the possibility that OST-PTP could dephosphorylate, i.e. inactivate, the insulin receptor in osteoblasts. An implication of this hypothesis is that insulin should be a positive regulator of osteocalcin bioactivity, in other words insulin signaling in osteoblasts should be necessary for whole-body glucose homeostasis in animals fed a normal diet.

Biochemical and genetic evidence gathered in cells and *in vivo* observations respectively demonstrated that indeed OST-PTP could dephosphorylate the insulin receptor in osteoblasts [27]. As inferred by these data and as hypothesized, mice lacking the *Insulin Receptor* only in osteoblasts displayed, when fed a normal chow, a decrease in circulating levels of the active form of osteocalcin, a decrease in insulin secretion, a glucose intolerance and an insulin resistance. Molecularly, insulin signaling in osteoblasts inhibits expression of *Osteoprotegerin (Opg)*, an inhibitor of osteoclast differentiation and function and as a result favors osteoclastic bone resorption. This is important because bone resorption is a process that requires an acidification of the bone extracellular matrix and a low pH is the only known means to decarboxylate proteins outside cells. This led us to show that the low pH of the resorption lacunae is necessary for decarboxylating and activating osteocalcin. Hence, insulin and osteocalcin are locked in a feed forward regulatory loop and insulin signaling in osteoblasts is necessary for whole-body glucose homeostasis in mice fed a normal chow [27, 28] (Fig. 1).

Pushing forward the logic of this novel regulatory axis taking place between bone and pancreas, one would expect that bone might contribute to whole-body insulin resistance in mice fed a high fat diet (HFD). As anticipated, in mice fed a HFD, bones become insulin resistant, this leads to a decrease in the circulating levels of the active form of osteocalcin and as a result, a decrease in insulin secretion and sensitivity [29]. Accordingly, mice lacking one copy of either the *Insulin receptor* in osteoblasts only or of *Osteocalcin*, even though they do not demonstrate any metabolic abnormalities when fed a normal diet, develop a more severe insulin resistance when fed a HFD due to a significantly lower circulating levels of the active form of osteocalcin compared to control mice fed the same HFD. Conversely, transgenic mice overexpressing the *Insulin receptor* in osteoblasts only are partially protected from whole-body insulin resistance when fed a HFD. At the molecular level, insulin resistance in osteoblasts develops because the increase in circulating levels of free saturated fatty acids it generates favors the ubiquitination of the insulin receptor in osteoblasts through the E3 ubiquitin ligase Smurf1. Thus these data further illustrate, but in a pathological condition, the importance of the osteocalcin in regulation of glucose homeostasis (Fig. 1).

6 Identification of Gprc6a as an osteocalcin receptor in mice and in humans

By and large, the majority of clinical studies that have been performed have shown a correlation between osteocalcin circulating levels and glucose homeostasis in humans as well [30–41]. These studies because of their correlative nature had to be interpreted cautiously and needed to have a more direct confirmation. This more direct confirmation could only come with the answer to the last question raised by the endocrine functions of osteocalcin, namely the identity of its receptor and of the signaling pathways it triggers in this various target cells.

Since this aspect of the work on the biology of osteocalcin does not belong to this review that focuses on the relationship between bone and pancreas, we will only briefly mention it here. Like most hormones, osteocalcin regulates several biological processes; one of them being the synthesis of testosterone by leydig cells in the testis and thereby male fertility [42]. Through the study of this particular aspect of osteocalcin biology we were able to identify a specific receptor for this hormone, a GPCR called Gprc6a [43]. Of note, the notion that Gprc6a might be a receptor for osteocalcin has been proposed by others [44]. *Gprc6a* is expressed in leydig cells of the testes and in β -cells of the pancreatic islets. Genetic evidence gathered in mice showed that *Gprc6a* is needed for osteocalcin regulation of insulin secretion and expression, and pancreatic β -cell proliferation [45] (Fig. 1) and that this function makes use of one particular transcription factor downstream of Gprc6a, CREB (unpublished data).

The male fertility phenotype of the *Osteocalcin*^{−/−} mice resembled rather closely a human condition called peripheral testicular failure [46–49]. This was an incentive to search for mutation either in *Osteocalcin* or in *Gprc6a* in patients affected by this disease. This search identified the same missense mutation in one of the transmembrane domains in two unrelated patients [50]. This mutation acts in cell-based assay and *in vivo* as a dominant negative mutation. Remarkably for the purpose of this review, both patients had an abnormal glucose tolerance. These results provided the first genetic evidence that GPRC6A is a receptor for osteocalcin in human as it is in mice. More importantly they provided evidence that osteocalcin fulfills the same endocrine functions in humans as it does in mice.

7 Perspective

As it is often the case in biology the data presented above raised more questions than they answered. The more specialized and immediate questions have to do with osteocalcin signaling in β -cell and with the mechanism whereby osteocalcin favors glucose utilization in peripheral tissues. A more general question although difficult to address is to understand the rationale for bone to regulate glucose homeostasis and the other functions it regulates. Attached to this latter question is the interrogation that has never been experimentally addressed until now of the functions of glucose itself in osteoblasts. If we now look beyond osteocalcin and if osteoblasts are to be bona fide endocrine cells then it is likely that they secrete more hormones than we know. Conceivably some of these as-yet unidentified hormones may regulate other aspects of energy metabolism. More generally, we as a field

will need to provide a verifying rationale for the existence of the endocrine function of the bone.

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Fig. 1. Regulation of glucose homeostasis by the bone-pancreas axis. Active osteocalcin is undercarboxylated osteocalcin and inactive osteocalcin is carboxylated osteocalcin