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Osteocalcin and the physiology of danger

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Abstract

Bone biology has long been driven by the question as to what molecules affect cell differentiation or the functions of bone. Exploring this issue has been an extraordinarily powerful way to improve our knowledge of bone development and physiology. More recently, a second question has emerged: does bone have other functions besides making bone? Addressing this conundrum revealed that the bone-derived hormone osteocalcin affects a surprisingly large number of organs and physiological processes, including acute stress response. This review will focus on this emerging aspect of bone biology taking osteocalcin as a case study and will show how classical and endocrine functions of bone help to define a new functional identity for this tissue.

Keywords

inter-organ communication; osteocalcin; response to danger; skeleton

Biologists who devote their careers to the study of a single organ usually pursue one of two questions. Granted, these questions can be asked in an innumerable number of ways. One question aims at unravelling the genetic, cellular and molecular mechanisms whereby an organ develops during embryogenesis. In a broad sense, this is the field of developmental biology for a given organ. Whether this is addressed through the study of stem cells, growth factors, and transcription factors, it asks the same fundamental question: how does organogenesis occur? A second question of organ biology aims at elucidating, also in genetic, cellular and molecular terms, how any given organ fulfils its functions; this is the field of molecular physiology. Addressing each of these questions has allowed the field of organ biology to make tremendous progress. It is however important to underscore, because it has biological implications, that the premises of those two questions of organ biology are profoundly different. In the case of organogenesis, the premise is that we do not know how cell differentiation occurs nor do we know the steps that lead to the organ's formation. In the case of molecular physiology, the implicit premise is different; it infers that we actually know what are the functions that this organ fulfils as if physiology was some kind of closed book, and what needs to be done is to define its molecular underpinnings [1-3].

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The genetic approach to organ biology has shown, and retrospectively it is not surprising, that this inference is erroneous and that a 'third dimension' of organ biology exists. This dimension relies on the premise that, as is the case for organogenesis, we in fact may not know all the functions that are fulfilled by a given organ. If one looks at the example of the white adipose tissue one may measure how correct, fertile and important this approach has been to organ and whole-organism physiology [4]. It is a tribute to the foresight of C. Bernard, W. Cannon and L.J. Henderson who put forward the founding concepts of whole-organism physiology, the one of milieu interieur, homeostasis and mutual dependence respectively, that their vision of physiology was validated experimentally, a century later by mouse genetics [5]. These three concepts and especially the one of mutual dependence implied that most organs talk to each other. In other words, that the study of organismal physiology may stopped short of its goal and that we may not yet know all functions of all organs. This is the agnostic approach that has added a new dimension to skeletal biology in the last 15 years.

The possibility of bone as an endocrine organ

The major reason to entertain the hypothesis that bone may have other functions besides making bone arises from the analysis of one rather peculiar cell biological feature of bone. Bone is the only tissue of the body that contains a cell type, the osteoclast, the main purpose of which is to actively destroy or resorb the host tissue, the mineralised bone extracellular matrix (ECM). This event occurs daily, in multiple locations, from birth to death [6-9]. It is as if bone had reinvented autoimmunity but, in this case, it is a physiological function or rather half of a physiological function since bone resorption is invariably followed by another energetically costly event, bone formation, which requires synthesis and secretion by osteoblasts of a large amount of collagen and other ECM components. During childhood, this perpetual alternation between bone resorption and formation contributes to longitudinal growth (bone modelling). It then continues throughout life, constantly renewing bone during adulthood (bone remodelling). Considered at face value, those are critically important physiological functions in the sense that they define the difference between life and death. Without bone modelling, there is no longitudinal growth, that is, no possibility to walk let alone to run, and therefore no way to survive in the wild. Hence, bone modelling is a survival function. As for bone remodelling [10,11], it prevents and repairs micro- and macro-damages, that is, fractures; in other words, it was for the longest time for mankind, the only orthopaedic surgeon around.

There is obviously a cost attached to each aspect of these functions. Both the destruction of bone in multiple locations and de novo bone formation are energetically costly. Furthermore, as one would expect, this cost is proportional to the surface covered by the organ, which is important because bones occupy a very large surface in our body. Although it is difficult to quantify the energetic cost of bone (re)modelling *in vivo*, the contention that it is an energetically costly process is supported by multiple clinical observations. A child who does not eat for any reasons will not grow, and adults who do not eat experience osteopenia and osteoporosis (low bone mass) [12-15]. In other words, the lack of energy intake brings bone modelling or remodelling to a stop. If one adds that gonadal failure at any point in life also invariably leads to bone loss, then this reading of bone biology and these

clinical observations suggest the hypothesis that there may be a coordinated control of bone (re)modelling, energy metabolism and reproduction that is endocrine in nature since the organs implicated are not next to each other [16]. We have explored and verified all facets of this hypothesis in the last 20 years [17]. The most far-reaching implication of this hypothesis, the one that is at the origin of the work presented in this review article, is that bone should be an endocrine organ that affects (aspects of) energy metabolism and reproduction [18,19].

How to define the hormonal nature of a protein?

It becomes now important to define what is a hormone: this definition is complex and based on several criteria. The first criterion is structural, as a rule of thumb if a protein is generated as a pre-pro-peptide, then cleaved in the cell synthesising it to generate a mature protein that is secreted in the general circulation, chances are that this protein may be a hormone [20-23]. The second criterion, one often neglected is its pattern of expression; most, hormones, such as leptin, insulin and PTH, though not all, are encoded by largely cell-specific genes [24-26]. A third criterion, one of the most important ones, is that the injection of this molecule in wild-type animals must up or (down)-regulate the physiological functions that this molecule is suspected to control. If this sufficiency criterion is not fulfilled, it is unlikely that this peptide is a hormone. The next criterion is the one of necessity, that is, that in mice or humans lacking signalling of this hormone, the physiological functions that it is supposed to regulate be hampered (or promoted). As a rule, which suffers only few exceptions, a hormone should be necessary and sufficient to regulate a given physiological function in an animal not challenged by any artificial manipulation, that is, as close as possible to conditions existing in the wild, when evolution invented this molecule. Fifth, this proposed hormone should have a receptor or receptor complex defined molecularly and functionally *in vivo*. The last criterion to be considered is whether the functions of this hormone are conserved in humans. Not all criteria are always met for a given protein but when they are, it is likely that the molecule under study is a hormone. Osteocalcin, the topic of this review article, fulfils all these criteria including the two most important: its injection affects in rodents and primates several signalling events and receptors for this hormone, three of them, have been identified.

Specific features of osteocalcin

Features of osteocalcin that are more specific to this molecule should be highlighted as they have influenced the work performed on this hormone. One of them distinguishes this hormone biochemically from most hormones, whereas two others, suggest that osteocalcin may have been invented by evolution as an endocrine means to sense and escape danger. The first feature of osteocalcin we highlight here is that once it is synthesised in osteoblasts and osteocytes, osteocalcin is carboxylated intracellularly on three glutamate residues by the enzyme gamma carboxylase [27-29]. This rare post-translational modification, the same that affects some coagulation factors, confers to osteocalcin a high affinity for hydroxyapatite, the mineral structure of the bone ECM [27,29]. This is why it was logically presumed when osteocalcin was first identified that it would be needed to initiate or regulate the mineralisation of the bone ECM. However, gain or loss of function experiments have

failed to detect any meaningful link between osteocalcin and ECM mineralisation in any organs [30,31]. Instead, the active form of the molecule is the uncarboxylated form of osteocalcin, which is undercarboxylated, on at least one glutamate residue, at position 17 in the mouse [18,32,33]. A second hallmark of osteocalcin is that the circulating levels of its bioactive form, surges in rodents and humans after aerobic exercise [34]. As explained later in this review, exploring the significance of this observation eventually brought to bear a redefinition of the purpose of the endocrine functions of bone. A third hallmark of osteocalcin is that its circulating levels decline steeply at midlife in all animal species tested from mouse to human [34]. This observation is important insofar as many of the physiological functions promoted by osteocalcin, that is, male fertility, memory, exercise capacity, also decline with age.

The foreseen functions of osteocalcin

In presenting the biology of osteocalcin, we will use a thematic rather than chronological presentation, because it better illustrates how a coherence slowly emerged from what seemed at first a hodgepodge of seemingly unrelated functions. What we qualify here as ‘foreseen’ functions of osteocalcin, are the ones inferred by the original hypothesis linking the regulation of bone mass, energy metabolism and reproduction.

The first endocrine function of osteocalcin that was described was its ability to enhance pancreatic β-cell proliferation, insulin synthesis, insulin secretion and energy expenditure [18,33,35]. This demonstration was achieved in mice and for several parameters in monkeys or isolated human pancreatic islets [36,37]. This was rapidly followed by the description of the ability of osteocalcin to signal in Leydig cells of the testes where it promotes, independently of the hypothalamo–pituitary–testes axis, the production of testosterone [19,38] (Fig. 1). The prominence of mouse genetics is such that we tend to lose of sight the fact that the ability of osteocalcin to increase pancreatic β-cell proliferation and mass, enhance insulin secretion by these cells and testosterone production by the Leydig cells of the testes was first established through gain-of-function experiments. Those consisted of either adding supernatant of wildtype or *Osteocalcin*$^{-/-}$ osteoblasts to cultures of pancreatic islets and Leydig cells, injecting exogenous osteocalcin or analysing a mouse model of osteocalcin gain-of-function, the *Esp*$^{-/-}$ mice [18,19]. It was also gain-of-function experiments that revealed that delivery of recombinant osteocalcin to mice challenged by a high-fat diet has beneficial effects on insulin secretion and glucose homeostasis [33,35]. It was also gain of function of experiments that showed that osteocalcin can increase insulin secretion in human islets [37]. The analysis of the *Osteocalcin*$^{-/-}$ mice established that osteocalcin is also necessary for glucose homeostasis and energy expenditure in animal fed a normal diet.

Several critical notions of bone biology arose from showing that osteocalcin favours insulin secretion by signalling in pancreatic β-cells. The first one was the demonstration that bone is a primary target of insulin signalling. Indeed, insulin signals back in osteoblasts to, if the tyrosine phosphatase osteotesticular phosphatase (ESP) does not degrade its receptor, increase bone mass accrual, trigger the secretion of bioactive osteocalcin and thereby contribute to whole-body glucose homeostasis [32] (Fig. 1). The necessity of insulin

signalling in osteoblasts for whole-body glucose homeostasis, is even more surprising in light of the observation that the disruption of insulin signalling in more classical insulin target organs, such as muscle and white adipose tissue, did not result in the anticipated glucose intolerance in animals fed a normal diet [39,40]. Arguably, these observations suggested that insulin might signal in other organs to maintain glucose homeostasis. In retrospect, bone is undeniably one of those organs. What was shown is that insulin signalling in osteoblasts favours bone resorption and therefore, the generation of undercarboxylated and bioactive osteocalcin. This occurs because the low pH that exists in resorption lacunae facilitates the removal of one or several carboxyl groups from osteocalcin and generates the active form of the molecule that then reaches the general circulation [32]. Accordingly, deleting the insulin receptor in osteoblasts results in a glucose intolerance phenotype in mice fed a normal diet. Conversely, diabetic animals develop insulin resistance in bone [41]. In contrast, when other tissues such as endothelial tissue, aberrantly adopt an osteogenic transcriptional programme they can cause deleterious effects on systemic glucose metabolism due to their pathological secretion of osteocalcin [42]. Another unanticipated benefit of showing that bone via osteocalcin favours insulin secretion was the demonstration that, independently of any hormone and because it is the main nutrient of osteoblasts, glucose may be the earliest molecular determinant of osteoblast differentiation, and a regulator of osteoblast functions postnatally [43].

If osteocalcin is a hormone, then what is its receptor in pancreatic β-cells and Leydig cells of the testes? The fact that when treating pancreatic islets or Leydig cells of the testes with osteocalcin, a bell shape curve in the response insulin or testosterone secretion respectively was observed [18,19,33] suggested that the receptor for osteocalcin might be a G-protein coupled receptor (GPCR). The reproductive functions of osteocalcin were more amenable to identifying a receptor than its metabolic ones simply because they affect only one sex. Hence, one could search for orphan GPCRs present in the male but not in the female gonad. Screening for orphan GPCRs expressed in Leydig cells of the testes but not in the ovary identified one of them, Gprc6a, that had been proposed by Quarles and colleagues to be an osteocalcin receptor a few years before [19,44,45]. The identification of Gprc6a as a possible receptor of osteocalcin in Leydig cells of the testes was a huge leap forward for the field in several ways. The most immediate one was the verification through cell-specific gene deletion experiments in the mouse that Gprc6a is required for osteocalcin signalling in Leydig cells in the mouse [19]. This was quickly followed by the genetic demonstration that Gprc6a is the receptor of osteocalcin in pancreatic β-cells as well [45,46]. Yet, probably the most critical consequence of the identification of Gprc6a as a receptor of osteocalcin has been to allow the study of the biology of osteocalcin in humans as will be described below. More broadly, it gave to osteocalcin a more complete hormonal identity, which has now been confirmed by numerous independent cell culture studies, *in vivo* studies in multiple species and multiple disease models, by correlative studies in humans and as we will describe, human genetic evidence [37,42,45-120]. It was also confirmed even more recently by the generation of a new genetic model of Osteocalcin deficiency [50]. While the evidence indicating that osteocalcin acts as a hormone in rodents and primates grows at an increasing pace, as the sample of references presented above indicates [37,42,45-120], this

does not mean that there are no studies in which *Osteocalcin*-deficient mice were presented as normal [51].

The reproductive phenotype of *Osteocalcin*$^{-/-}$ and *Gprc6a*$^{-/-}$ mice is a phenocopy of a human condition called peripheral testicular failure [121,122]. Like Osteocalcin$^{-/-}$ and Gprc6a$^{-/-}$ mice, affected patients experience infertility because of low circulating testosterone levels in the face of high circulating levels of luteinizing hormone (LH), the pituitary hormone that promotes testosterone secretion [19,38]. The very existence of this syndrome that develops in the absence of any chromosomal abnormalities or insult to the testes, inferred that an additional regulator of testosterone secretion may exist in humans. The similarity between *Osteocalcin* signalling-deficient mice and humans affected by testicular failure called for a molecular exploration of this disease. Sequencing *Osteocalcin* and *Gprc6a* in 59 of these patients identified, in two unrelated individuals the same missense mutation in GPRC6A [38]. This mutated allele acts as a dominant-negative one and inhibits the function of the wild-type allele. So too in this case the initial finding was rapidly followed by the identification of a polymorphism in GPRC6A rs2274911 + (Pro 91 Ser) that increases the risk of azoospermia more than four-fold in otherwise healthy men and is associated with insulin resistance in lean and obese subjects [123,124] (Fig. 1). On this basis and at least for energy metabolism and reproduction, the endocrine functions of osteocalcin appear to be conserved between rodents and humans. Subsequently, numerous gain-of-function studies including the identification of the KGKY human variant of the osteocalcin receptor, GPRC6A that is a gain-of-function in osteocalcin signalling have confirmed that it is the case [37,45,58,66,69,85,106,125,126]. In the case of the KGKY variant of the osteocalcin receptor, GPRC6A, which correlates with improved glucose metabolism in humans, it has been confirmed using genetic mouse models and treatment with exogenous osteocalcin that this human variant causes a gain-of-function in osteocalcin signalling and causatively improves glucose metabolism [58,79].

In closing, it is important to highlight that the regulation of energy metabolism by osteocalcin is not limited to its regulation of insulin secretion. Osteocalcin signalling through Gprc6a for instance is necessary for the regulation of hepatic energy metabolism and the secretion of FGF21 and for lipid metabolism in the adipose tissue and the liver [57,60,123,127]. The regulation of FGF21 expression in hepatocytes by osteocalcin is of interest for another reason: it furthers support the notion that osteocalcin is a regulator of regulatory molecules.

Moving away from the foreseen functions of osteocalcin

The hypothesis that there is a coordinated control, endocrine in nature, of bone growth/mass, energy metabolism and reproduction does not distinguish between energy usage in resting and exercising states for a given animal. Therefore, the subsequent identification of functions of osteocalcin that occur only during exercise did not invalidate the original hypothesis; it simply shifted it from one physiological state to another. In doing so, it revealed a coherence to all aspects of the biology of osteocalcin.

The ability to exercise is an evolutionarily conserved physiological function that in the wild, serves survival purposes: running away from predators and running towards food. More importantly, in modern societies, exercise provides numerous health benefits to sedentary people, that is, the majority of us living in developed countries [128-130]. This explains why the regulation of exercise is such an active topic of research. There is, of course, no aerobic exercise possible without the functional integrity of muscle. During exercise, myofibers of oxidative muscles, the ones most heavily used during endurance exercise, must take up large quantities of their nutrients, glucose and fatty acids (FAs), and catabolise them in order to generate energy in the form of ATP molecules [128-131]. The regulatory mechanisms involved in the regulation of muscle functions during exercise are still not well understood. Assuming that extracellular cues may regulate nutrient uptake and catabolism in muscle and adaptation to exercise, they could be either myokines, that is, molecules secreted by muscle during exercise that would boost nutrient uptake and catabolism in myofibers through various mechanisms [132-135] or hormones signalling in myofibers during exercise for the same purpose. The fact that during exercise muscles need to breakdown nutrients, that is, glucose and FAs de facto excluded insulin as a possible regulator of exercise since insulin is anabolic [136]. To make matters worse, circulating insulin levels decrease during aerobic exercise [136-138]. This led to a concept proposed 60 years ago of a humoral factor, or several, favouring exercise capacity in an insulin-independent manner [139].

Since circulating osteocalcin levels surge in mice and humans subjected to exercise, it is logical to ask whether osteocalcin is necessary for muscle function during exercise through the analysis of mice lacking Gprc6a exclusively in skeletal muscles ($Gprc6a_{Hsa}^{-/-}$) [34]. Osteocalcin signals in myofibers and to date the only known purpose of this signalling takes place during exercise, when circulating osteocalcin levels are increased [34]. Mice lacking osteocalcin or Gprc6a in myofibers only develop overtime a deficit in muscle function only during exercise and in exercise capacity. Importantly, the ability of osteocalcin signalling to increase muscle function during exercise is independent of muscle mass since it began to decrease at a time muscle mass is strictly normal [34,140]. To favour muscle function during exercise osteocalcin signalling in myofibers increases uptake and catabolism of glucose and FAs during exercise [75,83,91,140] (Fig. 2). In other words, even though osteocalcin is an insulin secretagogue, in at least one physiological setting, exercise, and in one organ, muscle, one of its functions opposes the anabolic function of insulin. Whether this has implications for insulin sensitivity in muscle remains to be addressed. Just as with insulin and testosterone secretion, osteocalcin signalling in myofibers is also sufficient to increase muscle function since a single injection of the hormone in young [3] wild-type mice increased the distance they could run on a treadmill by 25% [140]. Of greater biomedical importance is the fact that a similar injection in older [12-15] mice, whose circulating osteocalcin levels are low, conferred on them the ability to run for as long as 3-month-old mice [140]. This not only showed that osteocalcin is necessary and sufficient to increase exercise capacity; as it is for glucose homeostasis and male fertility, but that osteocalcin has anti-geronic properties, that is, it has the ability, when administered to older animals, to ameliorate consequences of aging [141].

Osteocalcin signalling in myofibers has at least one more function in myofibers: it increases during exercise, the expression and secretion in the general circulation of interleukin-6

(IL-6), the first myokine ever described [133,134,142]. IL-6 has been proposed to increase exercise capacity by enhancing liver gluconeogenesis, lipolysis and glucose uptake by myofibers [133]. The demonstration, through the analysis of mutant mouse strains lacking the osteocalcin receptor only in myoblasts, that *in vivo* osteocalcin signalling in myofibers accounts for most of the rise in circulating IL-6 levels during exercise, provided the opportunity to test *in vivo* whether IL-6 favours exercise through additional mechanisms. Together, the analyses of mice harbouring either a muscle-specific inactivation of IL-6, or a deletion of the IL-6 receptor in osteoblasts revealed that muscle-derived IL-6 is necessary for optimum ability to exercise and that it must signal in osteoblasts to enhance the secretion of osteocalcin to increase exercise capacity [36]. During exercise, osteocalcin and muscle-derived IL-6 are locked into a feed-forward regulatory loop in which the rise in circulating osteocalcin levels during exercise triggers the synthesis of IL-6; then IL-6 signals back into bone to increase the release of bioactive osteocalcin [36] (Fig. 2). Furthermore, the crosstalk between osteocalcin and IL-6 is conserved in humans since in individuals treated with monoclonal antibodies inhibiting IL-6 signalling, osteocalcin no longer increases in the blood stream after exercise [36]. In broader terms unravelling the regulation of adaptation to exercise by osteocalcin signalling in myofibers was a major clue towards revealing a coherence among all functions of osteocalcin. In addition, this work raised the question of the therapeutic relevance of this feed-forward loop between osteocalcin and IL-6 to combat the decrease in muscle function that characterises aging and many degenerative diseases.

A rupture

Even if it was not specifically included in the original hypothesis that links bone growth/mass, energy metabolism and reproduction in a triangular relationship, the ability of osteocalcin to up-regulate muscle function during exercise was a refinement. In contrast, the next functions represent a clear rupture from the original hypothesis. As such, they posed a conceptual challenge, which was to search for a coherence linking together the functions of osteocalcin.

Going back to basics, the word phenotype defines one or several physical characteristics immediately apparent to an observer in each animal then the *Osteocalcin*$^{-/-}$ mice have only one phenotype. Osteocalcin$^{-/-}$ mice are overtly, abnormally passive and docile. Not a single *Osteocalcin*$^{-/-}$ mouse ever escaped when placed on the top of a cage and no one ever reported to have been bitten by an *Osteocalcin*$^{-/-}$ mouse. That this docility exists in *Osteocalcin*$^{-/-}$ mice of both sexes indicated that it is not a mere consequence of the low circulating testosterone levels in male *Osteocalcin*$^{-/-}$ mice. Since this phenotype was so obvious, *Osteocalcin*$^{-/-}$ mice were subjected to a thorough behavioural analysis. To avoid the confounding factor that their lack of testosterone would represent this analysis was conducted in female mice [143]. This analysis revealed the existence of an increase in anxiety, a depression and a severe deficit in spatial memory in *Osteocalcin*$^{-/-}$ compared to wild-type littermates [143]. That these abnormal behaviours could be corrected through an intra-cerebro-ventricular infusion of *osteocalcin*$^{-/-}$ in *Osteocalcin*$^{-/-}$ mice, at a dose that did not cross the blood brain barrier, indicated that osteocalcin signals within the brain [143]. Accordingly, using *Osteocalcin*$^{-/-}$ mice as a tool, it was possible to show that osteocalcin crosses the blood-brain barrier, accumulates in specific areas of the brain, binds

to and affects the activity of neurons in the dorsal and median raphe nuclei, the ventral tegmental area and the CA3 region of the hippocampus, promotes the synthesis of all monoamine neurotransmitters and of BDNF and inhibits the one of GABA, an inhibitory neurotransmitter [143] (Fig. 3). These functions of osteocalcin in the brain were confirmed in gain-of-function experiments [76]. Subsequently, clinical investigations correlating brain structure with circulating osteocalcin levels have suggested that circulating osteocalcin levels correlate with cognitive performances in humans too [67,144].

Osteocalcin is not readily detectable in the general circulation of mouse embryos before E15.5 [46,143,145]. Furthermore, the phenotypes described above were all observed in adult mice [143]. How then could one explain the existence of an anatomical defect in the brain of newborn *Osteocalcin*$^{-/-}$ mice, and even of *Osteocalcin*$^{-/-}$ embryos (smaller brain, frequent absence of the corpus callosum, small and dysmorphic hippocampus)? Addressing this conundrum revealed that maternally derived osteocalcin begins to cross the placenta at E14.5, and can be found in the general circulation of *Osteocalcin*$^{-/-}$ embryos that are carried by *Osteocalcin*$^{+/-}$ mother [143]. During embryogenesis, maternally derived osteocalcin reaches the brain, and prevents neuronal apoptosis in the hippocampus thereby contributing to the establishment of spatial memory in adult mice [143]. This observation along with the ability of osteocalcin to regulate glucose homeostasis certainly suggests that the dysregulation of the synthesis of this hormone may contribute to the maternal influence on the metabolic and psychological health of the offspring [146]. Of course, these observations do not mean that bone is more important than the brain when it comes to controlling anxiety, depression or cognition. Rather they say that no organ, not even the brain, is an island that lives and functions in a state of splendid isolation; as inferred by the concept of mutual dependence put forward by L.J. Henderson [5], no physiological process is the function of only one organ.

Precisely because it is made in bone, and affects the brain, making a convincing argument that osteocalcin is a determinant of anxiety, depression and memory needed the identification of a receptor. This became a problem when it became clear that *Gprc6a*, the only known receptor of osteocalcin at that time, is not expressed in any of the brain regions where osteocalcin signals. To make matters worse, *Gprc6a*$^{-/-}$ mice have normal spatial memory and are neither depressed nor anxious [143]. Thus, it became likely that osteocalcin had a second receptor that was expressed in the brain. Searching for orphan GPCRs of the same subfamily as Gprc6a, the class C subfamily, identified Gpr158 as a candidate receptor for osteocalcin in the hippocampus and the ventral tegmental area. Molecular, electrophysiological and behavioural analyses of mice lacking *Gpr158* only in neurons and of compound heterozygous mice lacking one allele of osteocalcin and one allele of *Gpr158* only in neurons, demonstrated that osteocalcin signals in specific regions of the brain through Gpr158 to control the synthesis of neurotransmitters, anxiety, depression and hippocampal memory [147] (Fig. 3). Again, gain of function experiments using viral vectors or injections to increase osteocalcin levels *in vivo*, verified the importance of Gpr158 for osteocalcin signalling in the brain [76].

The role of osteocalcin in the brain does not stop there. Indeed, an even more recent investigations it was shown that osteocalcin inhibits axon myelination. This work identified

a third osteocalcin receptor, Gpr37, that is found in the brain as mediating this latter function of osteocalcin. Loss-of-function and pharmacological gain-of-function experiments showed that osteocalcin signals through Gpr37 in oligodendrocytes to inhibit oligodendrocyte myelination and differentiation. As a result, mice lacking osteocalcin signalling demonstrate a diminished pain sense reflex, a phenotype that may be explained in part by increase myelination and slower neuronal conductance in afferent neuronal circuits [50]. Future work will be needed to determine whether Gpr37 interacts with Gpr158 in the central nervous system and what other functions osteocalcin fulfils by signalling through Gpr37. This work is also important for other reasons. A first is that it uses its own model of *Osteocalcin* deficiency, a second reason is that these authors confirmed that osteocalcin signals through Gprc6a and Gpr158 [50].

In the absence of any human genetic data (to date no homozygous loss-of-function mutations in *Gpr158* have been identified in humans), how could one make the case of the importance of the regulation of brain functions such as cognition and anxiety by osteocalcin? To do so we took advantage of a hallmark of osteocalcin and a previously published study that had, rightly so, electrified the field of aging biology [141]. This hallmark of osteocalcin is that circulating osteocalcin levels plummet with age, just like memory does. The finding was that injections in old mice of plasma from young mice restored in the former a level of spatial memory or cognition only seen in young mice. However, when the plasma of young mice used in this experiment was obtained not from wildtype but from *Osteocalcin*$^{-/-}$ mice, the beneficial effect on cognition and anxiety in older mice was gone. This beneficial effect was also lost when the plasma of young WT mice had been depleted of osteocalcin prior to being injected in old mice [147]. Conversely, this rejuvenating ability was restored to the plasma of *Osteocalcin*$^{-/-}$ mice by simply adding osteocalcin into it. The latter two experiments ruled out that the pro-cognitive function of osteocalcin in wildtype mice was of developmental origin. Taken together these experiments identified osteocalcin as an indispensable contributor of the beneficial impact of plasma from young mice on the cognition of older mice [147]. Consistent with this notion, delivering osteocalcin chronically for 2 months in 16-month-old wild-type mice restored in them cognitive functions seen only in young wildtype mice. Thus, in the brain like in muscle, osteocalcin is a bona fide anti-geronic molecule.

Towards a coherence

Glucose metabolism, male fertility, exercise capacity, memory: why would a single hormone regulate these seemingly disparate physiological processes? And why would this hormone be made by bone cells? To be fair, when analysed in a modern context, there is no obvious a common thread linking together the different functions of osteocalcin. However, when these functions are placed in an evolutionary context, the emerging picture has more coherence. For instance, the ability to exercise that is so significantly enhanced by osteocalcin was invented by evolution as a tool to escape danger when living in a hostile environment such as the wild. The mobilisation of glucose that is also enhanced by osteocalcin is needed for exercise, in other words to escape danger. As for memory, it was needed to remember where food and/or the predators were an hour or a day ago. Lastly, in the case of acute danger, circulating testosterone levels tend to increase [148]. In this light osteocalcin

acquires a novel identity, the one of an hormone that allows bony vertebrates to up-regulate physiological functions needed to escape acute danger. Thus, osteocalcin may contribute to defining an endocrinology of danger. In broader terms, if their survival purpose is what gives a coherence to the endocrine functions of bone, is this specific to these functions or is it a general feature of bone physiology? The latter hypothesis is supported by the fact that bones are hardened structures that likely evolved to protecting internal organs from injuries in the case of trauma or predation [149]. Further, the bones present in the middle ear are needed to hear, which is a sense that is absolutely needed to detect danger. Lastly, bones are needed to move and run, that is, to escape danger. Finally, the rib cage is required for respiration and its functionality during exercise. Thus we hypothesize that both the classical and endocrine functions of bone contribute to the organismal response to danger.

The hypothesis that osteocalcin may be needed to upregulate the response of a living animal to an acute danger, raises the question as to whether it regulates other physiological function(s) needed to escape an acute danger? The acute stress response serves to maintain or restore homeostasis in animals facing an acute danger [150-152]. The sympathetic nervous system, through its release of catecholamine in peripheral organs, is the ultimate mediator of the acute stress response. However, what is unknown is how the sympathetic nervous system becomes activated during the acute stress response Could this be an endocrine signal? In essence, a stress hormone is defined by two criteria. First, its circulating levels increase during an acute stress response and second, it should up-regulate physiological processes that are recruited during the acute stress response. We tested whether osteocalcin fulfils any of these two criteria.

What was first observed in mice and in humans, is that circulating osteocalcin levels jump up to four-fold within 2 min following exposure to stressors [153]. For this to happen, stressors must signal in the basolateral amygdala region of the brain and that the neurotransmitter glutamate links the brain to the skeleton [153]. When released during an acute stress response by glutamatergic axons abutting osteoblasts, glutamate enters into osteoblasts through the transporter Eaat1 (also termed Glast) [154,155] to act as a competitive inhibitor of the gamma carboxylase enzyme that is responsible for the carboxylation and inactivation of osteocalcin [153]. As a result, undercarboxylated osteocalcin, is released from osteoblasts during the acute stress response [153]. Osteocalcin also fulfils the second criterion of a stress hormone. Mice lacking osteocalcin or its receptor in peripheral organs are unable to mount a full acute stress response when exposed to stressors [153]. *Gprc6a* is expressed in post-ganglionic parasympathetic neurons but not in sympathetic neurons; molecular and electrophysiological studies performed in mice or neurons either WT or lacking Gprc6a showed that osteocalcin signalling in parasympathetic neurons normally inhibits the synthesis, and recycling of acetylcholine (ACH) [156] and its release [153]. This leaves the sympathetic tone unabated and able to drive the acute stress response. Similar results were obtained in human tissues [153,157]. These results identified osteocalcin as a bona fide stress hormone needed for a full acute stress response [153]. And here again, osteocalcin acts upstream of another regulatory molecule, ACH.

But this left an elephant in the room untouched: what about the surge in circulating glucocorticoid levels that occurs during the acute stress response? Is it needed to initiate this

process? These questions were addressed in mice and rats that had been adrenalectomised (ADX), and in human patients unable to produce cortisol [153,157]. Remarkably, stressors in these ADX animals and adrenal insufficient human beings could trigger a normal acute stress response, indicating that this response needs neither adrenal steroid hormones nor the catecholamines produced by the adrenal glands [153]. Another observation of great importance made in ADX animals is that, consonant with the inhibition of *Osteocalcin* expression by glucocorticoid hormones [158], circulating osteocalcin levels were higher at baseline and rose to higher levels after exposure to stressors than they did in sham-operated animals [153]. The reason ADX animals can mount a normal acute stress response is because they are hyperosteocalcinemic. In other words and at least in ADX animals, osteocalcin signalling in post-ganglionic parasympathetic neurons is sufficient to initiate the acute stress response in order to escape danger [153].

Put together, the inhibition of *Osteocalcin* expression by glucocorticoid hormones and the role of osteocalcin in the acute stress response suggested another hypothesis, another function of osteocalcin and the skeleton. Could it be that this regulation of *Osteocalcin* expression infers that in fact osteocalcin is a previously unappreciated regulator of glucocorticoid biosynthesis? In agreement with this hypothesis, increasing acutely or chronically circulating osteocalcin levels is sufficient not only in rodents but also in non-human primates, to increase glucocorticoid hormones circulating levels. Remarkably, either one of these two manipulations was also sufficient to increase the circulating levels of aldosterone the mineralocorticoid hormone synthesised by cells of the glomerulosa layer in the cortex of adrenal glands. These results suggested that osteocalcin might be a regulator of adrenal steroidogenesis, that is, glucocorticoid and mineralocorticoid hormones biosynthesis [159]. Given the complexity of the regulation of adrenal steroidogenesis that involves numerous molecules and multiple organs addressing this question required first to determine where is the receptor of osteocalcin expressed along the hypothalamo–pituitary–adrenal (HPA) axis or the renin–angiotensin system (RAS)? What this analysis showed is that one of the three receptors of osteocalcin, Gpr158, is expressed in the adrenal cortex and in no other relevant tissues, and that mice lacking Gpr158 in all cells or in cells of adrenal cortex develop an adrenal insufficiency characterised by low circulating corticosterone and aldosterone, high blood potassium concentration, and lower blood pressure. Hence, osteocalcin signalling in the adrenal glands is not only sufficient to promote adrenal steroidogenesis, it is also necessary for this process, even in the presence of a functional HPA axis and RAS [159].

Unexpectedly, the pool of osteocalcin that is needed to favour adrenal steroidogenesis is neither the maternal nor the postnatal one but rather the embryonic one. Independently of the presence of a functional HPA axis, embryonic osteocalcin is necessary to maintain the expression of the master regulator adrenal development, *steroidogenic factor 1* or Sf1 [160], beyond E14.5 in the prospective adrenal gland. As a result of this function, in the absence of embryonic osteocalcin differentiation of cells of the adrenal cortex is hampered and the number of these cells in the adrenal cortex is significantly decreased during embryonic development and throughout life. Remarkably, the absence of osteocalcin only after birth does not affect adrenal steroidogenesis, indicating that this is a specific function or set of functions of embryonic osteocalcin [159]. This most recent work not only adds credence to

the notion that osteocalcin may contribute to the response of bony vertebrates to an acute danger, suggests that many cross-talks between organs remain to be discovered, but it also reveals that embryonic osteocalcin, which for a long time was not considered as biologically important, has its own set of functions. These developmental functions certainly determine in part adrenal development but beyond embryogenesis they also affect parameters of homeostasis that postnatal osteocalcin does not regulate.

Can we summarise what we learnt, propose where we could be going?

Since the work on osteocalcin biology and relevance is only beginning, we can only be brief, otherwise we run the risk of being wrong in the long run. Bringing a coherence to the functions of osteocalcin requires to walk the walk, namely to describe, as exhaustively as possible, what the functions of osteocalcin are. This exploration is not over, yet it has already validated the original premise of the work, which is that bone has functions other than making bone in rodents and primates. But, as one should have expected, this exploration revealed that osteocalcin has more functions than inferred by the original working hypothesis.

A first lesson learnt from this work is that with the appearance of bone during evolution came novel ways to regulate several physiological functions such as glucose homeostasis, male fertility, exercise capacity, cognitive functions and the acute stress response. That many of the physiological functions regulated by osteocalcin are needed to escape acute danger suggests that this hormone and by extension bone may be defined in part by an endocrinology of danger. Since many of these physiological functions decline with age, these regulatory roles of osteocalcin may pave the way to new and adapted therapies for several age-related and degenerative diseases.

In the short term, there are implications for osteocalcin and bone biology as a whole. A general feature of osteocalcin that emerges more and more clearly from this body of work is that for many of its regulatory functions it appears to act indirectly, that is, by recruiting other regulatory molecules that actually do its job. Osteocalcin for instance does not regulate memory on its own, rather it recruits a series of neurotransmitters that promote memory. Likewise, osteocalcin regulates glucose homeostasis in part by being upstream of insulin and in part by having functions opposed to the one of insulin, for example, it favours liver gluconeogenesis. The clearest example of this role of regulator of regulatory molecules resides in the regulation of testes and adrenal endocrine functions by osteocalcin. In that respect, one cannot avoid noticing that osteocalcin is necessary for the proper synthesis of three steroid hormones in two different organs. More broadly, what is unusual is the sheer number of regulatory molecules the secretion of which is regulated by osteocalcin. This peculiarity of osteocalcin suggests that evolution has endowed bone with an unusually high number of regulatory functions. Although one cannot claim that we fully understand why all these functions are regulated by osteocalcin, the regulatory functions exerted by this hormone find, at least for several of them, a coherence, if not a common purpose, when placed in the context of the biological response to danger. The notion that osteocalcin may be a hormone orchestrating the response to danger does not exclude other ways to rationalise all its functions nor does it exclude the possibility that other hormones do contribute to

the response to danger. It simply provides a tool to look for other endocrine functions of osteocalcin and reconcile the endocrine and the structural functions of bone. It provides a working hypothesis to identify novel physiological functions regulated by bone-specific secreted molecules.

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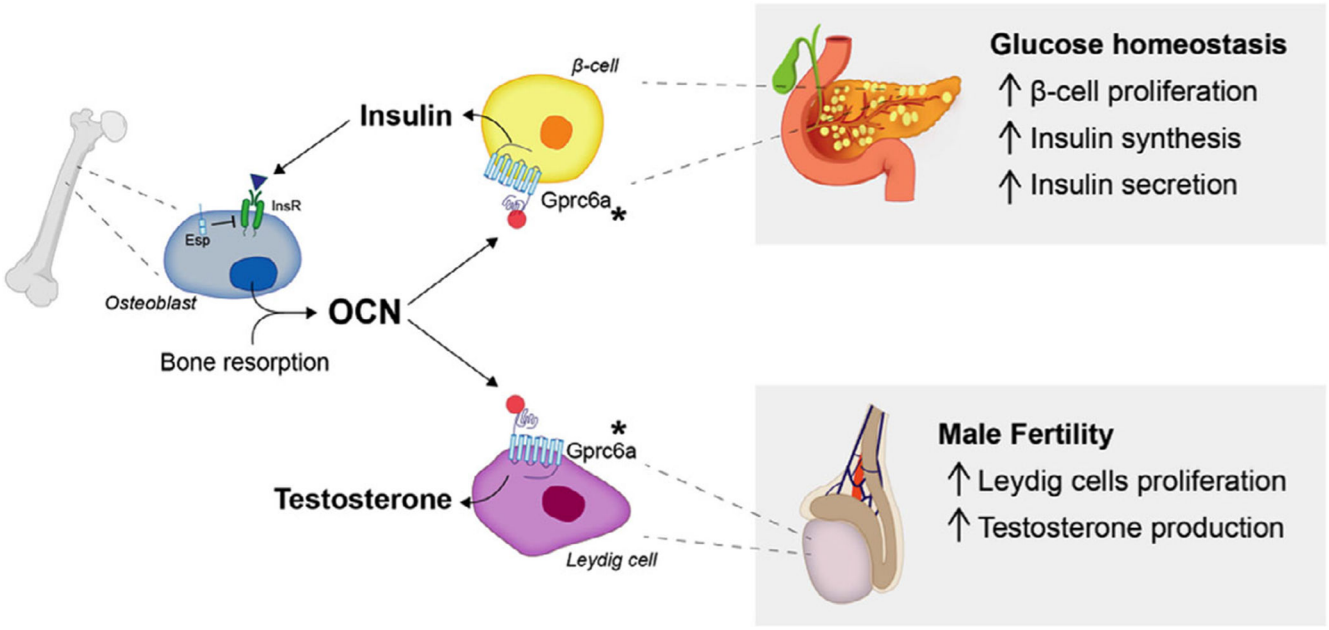

Fig. 1.

Coordinated endocrine control of bone fertility and energy metabolism. Schematic showing the effect of osteocalcin (OCN, red circle) on Leydig cells and β-cells and the feedback loop in which insulin regulates OCN release from bone. OCN receptor Gprc6a and Insulin receptor are schematically depicted in light blue and green respectively.

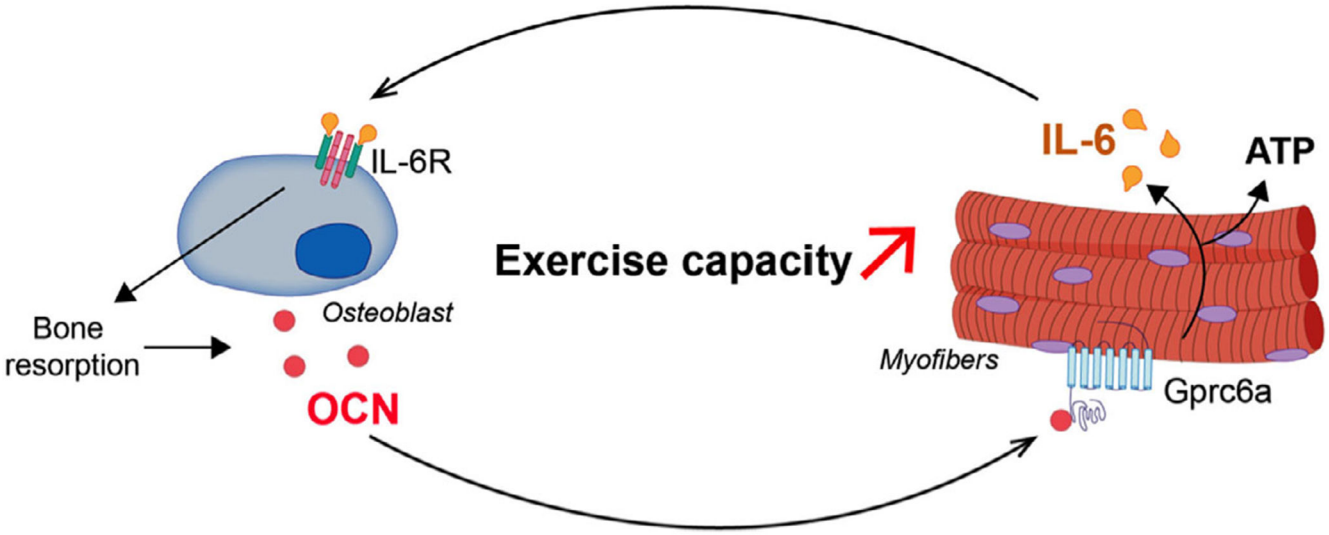

Fig. 2.

An endocrine loop between bone and muscle that increases exercise capacity. Schematic depicting the effect of osteocalcin (OCN) on myofibers and the effect of muscle-derived IL-6 (orange teardrops) on OCN (red circles) release from bone. During exercise, IL-6 acts on osteoblasts to increase the expression of RankL, the primary regulator of osteoclast differentiation and thus bone resorption. IL-6R (IL-6 receptor) and OCN receptor (Gprc6a) are schematically depicted. ATP indicates adenosine triphosphate.

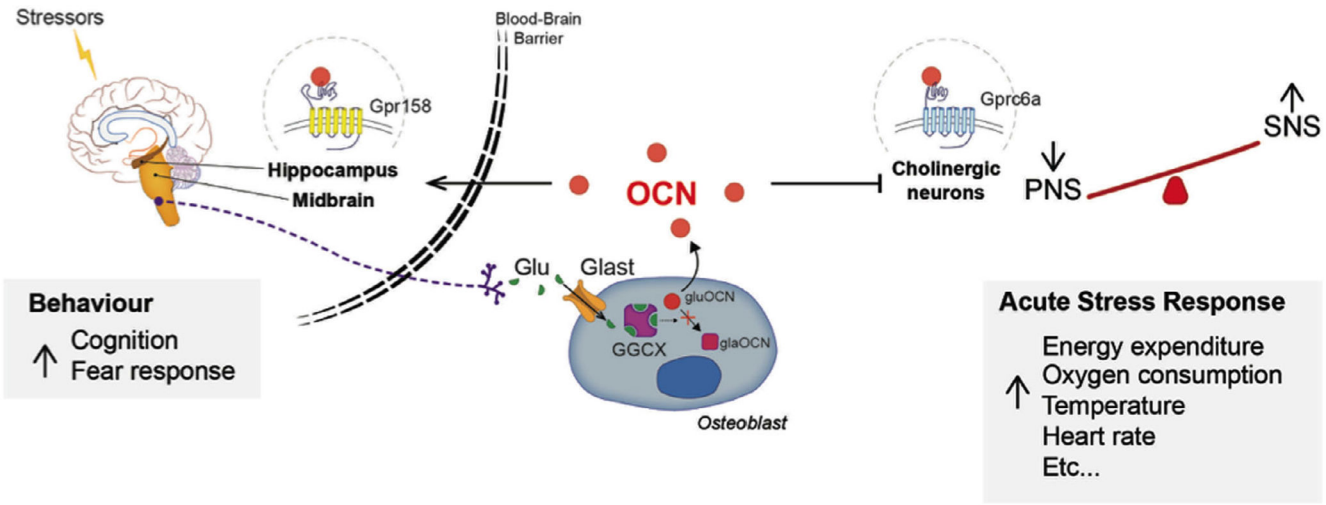

Fig. 3.

An endocrine system that promotes survival in response to danger. Schematic depicting the release of osteocalcin (OCN) during acute stress and its effect on parasympathetic neuron activity. Glu indicates glutamate, gluOCN indicated bioactive osteocalcin, glaOCN indicates inactive osteocalcin, PNS indicates parasympathetic nervous system and SNS indicates sympathetic nervous system.