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Visions & Reflections (Minireview)

Osteocrin – Beyond just another bone protein?

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What is osteocrin and how does it function?

Our group originally identified osteocrin (Ostn) as a secreted molecule expressed in bone forming cells, the osteoblasts [1]. *In situ* hybridization showed Ostn expression was highest in the osteoblasts of cortical bones in embryonic mice, leading us to ascribe Ostn as bone specific. However, we [2] and other groups [3, 4] have subsequently detected its expression in other tissues of mesenchymal origin such as muscle. Functional testing of Ostn *in vitro* showed that conditioned media from HEK293 cells overexpressing full-length Ostn could inhibit the differentiation of primary osteoblastic cultures [1]. However, it was not clear whether the full-length form of Ostn was the active molecule. Full-length Ostn gives rise to a 103-amino acid secreted product upon cleavage of its signal peptide [1]. When overexpressed and secreted from HEK293 cells, Ostn is further processed generating smaller peptide fragments. Inspection of the Ostn peptidic sequence revealed the presence of dibasic sites, one of which (KKKR), represented a putative furin recognition motif (Fig. 1A) and mutation of this site abolished fragmentation. Other potential cleavage sites are also present within Ostn and whether they represent *bona fide* sites for furin or other convertases is still unknown. The presence of the KKKR site, frequently associated with peptide hormones, led us to hypothesize that Ostn itself could be a hormone-like molecule. This was further supported by the fact that Ostn was independently identified in a search for peptide hormones in the human proteome based on a bioinformatics approach [5]. Intriguingly, the C-terminal fragment generated by cleavage at the KKKR site comprises two domains with homology to the natriuretic peptides (NPs), specifically in the region matching the ring structure of the NPs, where the residues demonstrated to be essential for binding of NPs to their receptors are highly conserved (Fig. 1A) [2]. The natriuretic system consists of three related NPs (ANP, BNP and CNP), and three receptors mediating the biological activity of these peptides [6]. The GC-A receptor, which preferentially binds ANP and BNP, and the GC-B receptor, whose cognate ligand is CNP, are coupled to guanylyl cyclase, producing cGMP as a secondary messenger. The third receptor, natriuretic peptide clearance receptor (NPR-C), has no guanylyl cyclase activity and binds all three NPs with similar affinity. To date, no specific endogenous specific ligand has been identified for NPR-C, and it is thought to act mainly as a clearance receptor, although other roles have been proposed [7]. We therefore hypothesized that cleavage of Ostn

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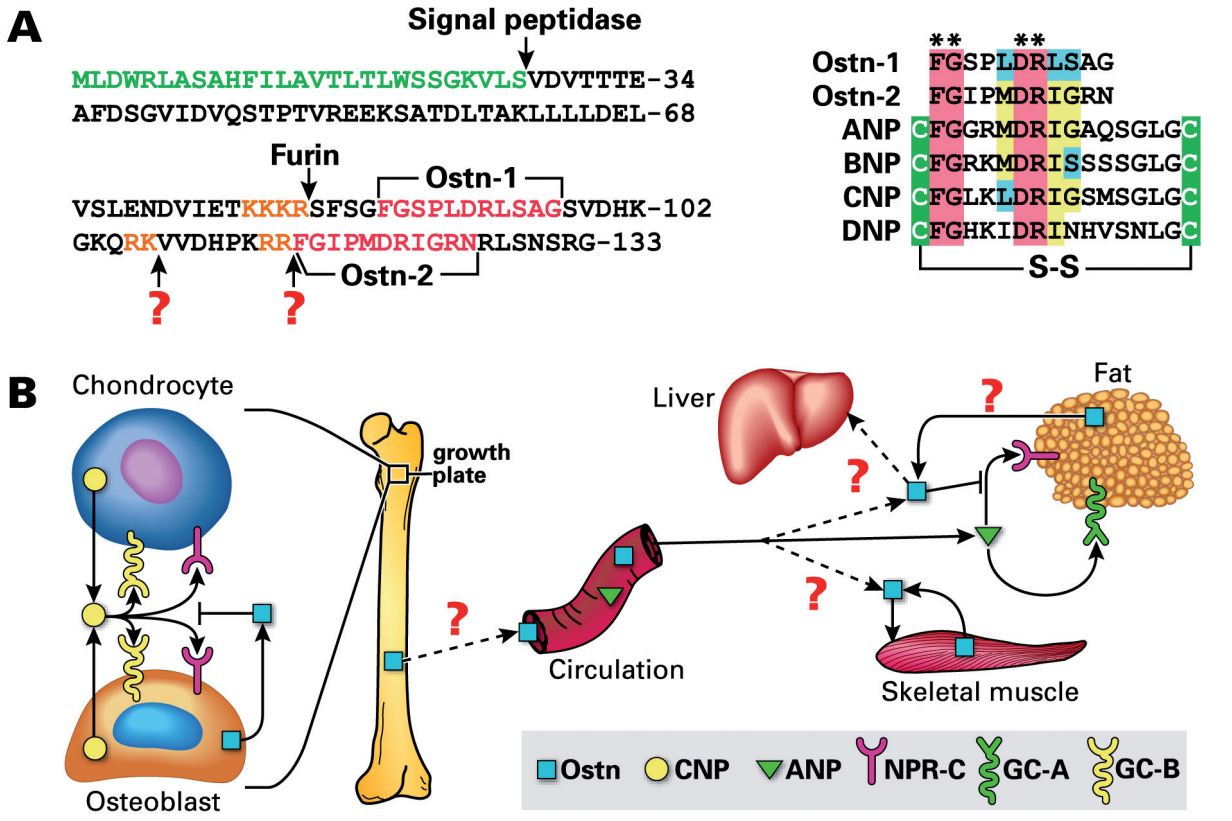

Figure 1. Osteocrin (Ostn) is a small secreted protein with homology to the natriuretic peptides (NPs) and binds the natriuretic clearance receptor (NPR-C). (*A*) The human Ostn sequence is shown at left with some of the salient features highlighted: cleavable signal peptide (green), the 'dibasic' sites (orange), and the two domains having similarity to the NPs (red). These features are highly conserved between Ostn from all species examined [2]. On the right the alignment of Ostn NP motifs with the human NPs (ANP, BNP, and CNP) is shown along with dendroaspis natriuretic peptide (DNP) that is found in the green mamba venom. For simplicity, only the cyclical portion of the NPs is presented with the conserved and similar residues highlighted. The asterisks indicate residues deemed most important for binding to the NPR-C. (*B*) Cartoon illustrating the verified and hypothesized roles for Ostn. Ostn has been shown to be expressed at least in osteoblast, muscle, and fat. Ostn is a specific ligand of the NPR-C and in bone it modulates CNP actions within the growth plate chondrocytes to accrue long bone growth. The functional consequences of the CNP/GC-B/NPR-C axis on osteoblasts are still unknown. In skeletal muscle, Ostn gene expression is up-regulated by fasting, through at least an insulin-dependent pathway. Secreted Ostn is believed to attenuate glucose transport and glycogen synthesis but the exact mechanism remains undefined. It remains unknown as to whether Ostn is present in the circulation, where its primary site of production would be, and if it can act on distant organs that express NPR-C. In fat, for instance, Ostn could favor lypolysis indirectly through diminished local clearance of ANP by NPR-C.

might generate active peptides that could interact with the natriuretic system. We subsequently showed that the NP motifs within Ostn were functional, and that Ostn selectively binds to the NPR-C, but not to the guanylate cyclase receptors GC-A and GC-B [2]. By binding to NPR-C, Ostn, as either the whole protein or peptidic fragments comprising the NP motifs, is able to modulate the response of cells to CNP or ANP by presumably increasing their availability to bind to their respective cognate receptors GC-B and GC-A.

Does Ostn have a function in the skeleton?

The original finding that Ostn is expressed in osteoblasts and that treatment of osteoblasts with Ostn inhibits their differentiation led us to hypothesize a role for Ostn in the skeleton. We therefore generated a transgenic mouse model overexpressing Ostn in osteoblasts driven by a collagen type I promoter. The Ostn-transgenic animals displayed elongated long bones with significantly elevated bone cGMP levels, the second messenger generated after activation of the GC-B [2]. We therefore proposed that Ostn, through blocking the clearance activity of NPR-C, increases CNP levels, thereby increasing GC-B activation, which in turn stimulates proliferation/differentiation of growth plate chondrocytes (Fig. 1B). More importantly, these results identified Ostn as the first example of a naturally occurring specific ligand for the NPR-C. The significance of the CNP/GC-B/NPR-C axis in longitudinal bone growth is now widely recognized. Several animal models have been generated in which each member of the pathway has been disrupted or

overexpressed with dramatic consequences on long bone growth (reviewed in [8–10]). These models illustrate the primary importance of CNP signaling in postnatal endochondral ossification whereby mineralized bone is formed through a complex series of steps arising from a pre-formed cartilaginous template. They have provided unique opportunities to unravel the actions of CNP in bone and its molecular mechanisms of action and also identified a new therapeutic approach for various forms of chondrodysplasia. For instance, a transgenic model overexpressing CNP in growth plate chondrocytes was shown to be able to rescue the achondroplasia phenotype of the FGFR3-activating mutation through the MAPK pathway [11]. Due to the actions of Ostn as a modulator of CNP availability, it is possible that Ostn overexpression in osteoblasts could also rescue the FGFR3 mice through a similar mechanism. The ability of Ostn produced from distant osteoblasts to diffuse far enough into the growth plate to affect chondrocytes is supported by our Ostn transgenic mouse model [2]. A number of human syndromes displaying dwarfism or bone overgrowth due to growth-plate effects have also been reported to be the consequences of mutations in either CNP or GC-B [12–14].

Another possibility to explain the effects of Ostn is that NPR-C could also signal inside the cell and not serve simply as a clearance receptor [7]. It has recently been shown that CNP-mediated signaling in cultured rat osteoblasts is mediated through NPR-C and a pertussis toxin-sensitive phospholipase C pathway [15]. In bone explant cultures, however, it was shown that a linear analogue specific for the NPR-C was able to accentuate CNP effects from normal but not from CNP-depleted mice, therefore suggesting the NPR-C does not appear to signal directly in chondrocytes [16].

Other existing models may also be useful to help demonstrate a possible role for Ostn in the skeleton. One is the "megabladder" mouse [17, 18] in which embryonic expression of Ostn was found to be elevated fourfold as a result of a duplication of a locus on chromosome 16 containing the Ostn gene. The developmental bladder defect of the megabladder mouse, however, is not caused by Ostn but to the overexpression of a neighboring gene called urotensin II-related peptide. Although no gross whole body change have been reported in this mouse model, it would be interesting to investigate whether they have subtle abnormalities of the skeleton. Further support for a role of Ostn in bone growth comes from the positive association between shank length and the Ostn locus in chickens [19].

The phenotypes described above are all thought to be mediated through the growth plate chondrocytes. However, both the CNP/GC-B/NPR-C axis and Ostn, are also expressed in osteoblastic cells [1, 15, 20–22]. The contribution of osteoblasts to the bone defects of the various models described above has not been considered to play a key role because of the lack of effect on intramembranous bones such as the calvaria and mandible. These bones are formed directly through the differentiation of mesenchymal progenitors into osteoblasts without an intermediate chondrocyte stage. However, direct effects of both CNP and Ostn on osteoblasts *in vitro* have been reported with Ostn inhibiting [1] and CNP stimulating [15, 20–22] the mature osteoblast phenotype. The reason for this difference is not clear but may reflect an *in vitro* artifact due to the isolation of specific cell types and/or differing peptide affinities. Interestingly and somewhat surprisingly the Ostn transgenic [2] and the CNP-knockout [23] and -overexpressing mice [11] did not show an appreciable osteoblast phenotype *in vivo*, possibly reflecting differences in either localized peptide levels or interactions with other tissues/cell types, between an *in vitro* cell model and a mouse. A transgenic mouse overexpressing CNP in osteoblasts has recently been generated [13] and these mice exhibit an even more dramatic long bone overgrowth than the previous model of chondrocyte-specific CNP overexpression [11]. Whether the action of CNP in this model is mediated through paracrine effects on chondrocytes or in an autocrine fashion on osteoblasts (or both) is unknown. Irrespective of the mechanism involved, this lends support to the possibility that peptides derived from osteoblasts (such as Ostn) can diffuse far enough into the growth plate to locally modulate CNP levels and effect chondrocyte activity. However, at present, a direct role for Ostn or the natriuretic system in osteoblast remains to be unequivocally demonstrated *in vivo*.

What is the role of Ostn in other mesenchymal tissues?

Shortly after our initial characterization of Ostn in bone cells, the same gene was discovered in muscle and termed musclin by that group [3]. We have subsequently confirmed this observation and also detected Ostn in tendons and ligaments [2]. This highlights an intriguing possibility that perhaps Ostn is a marker of mesenchymal cells and may play a role in mediating effects of the natriuretic system in all these cells. Banzet et al. [4] further refined the expression profile of Ostn in muscle and found it is highest in muscle fibers that contract more rapidly and favor glycolytic metabolism (type IIB fibers), and low or absent in slow-twitch fibers. More importantly, Ostn

expression in muscle cells is regulated by nutritional status [3], with Ostn dramatically down-regulated by fasting, and returning to control levels after re-feeding. Furthermore, insulin was demonstrated to be a potent regulator of Ostn expression in C2C12 myotubes with the secreted Ostn processed at the KKKR site as in HEK293 cells [2]. Functionally, recombinant Ostn inhibited glucose uptake and glycogen synthesis by C2C12 cells, but the mechanism(s) involved remain to be determined. The same group more recently showed that insulin-increased Ostn expression was dependent on the PI3K/AKT1 pathway [24]. One of the possible downstream transcriptional effectors was Foxo1, whose expression is reciprocal to that of Ostn during fasting/re-fed regimen, and is a target of AKT1 upon insulin stimulation. Overexpression of a non-phosphorylated constitutively active form of Foxo1 was shown to repress Ostn expression in C2C12 cells. Interestingly, *in vivo* models with altered muscle gene expression also present changes in Ostn expression in muscles [24, 25]. These data convincingly suggest that Ostn in muscle is regulated by metabolic status, but how Ostn ties into a general scheme of energy handling is unclear. Further, a role for Ostn interaction with the natriuretic system in muscle has not yet been addressed.

Ostn has also been found to be expressed in white abdominal fat [3]. In fat, lipolysis is reciprocally regulated by insulin and the ANP/GC-A/NPR-C system or adrenergic signaling [26, 27]. The net effect ANP has on lipolysis, however, is dictated by the relative ratios of expression of NPR-C and GC-A. Hence, it appears only operative in primates where GC-A levels exceed those of NPR-C, but not in lower species such as rodents where NPR-C predominates. This blockade of NPR-C may have dramatic consequences on fat mass. In accordance with this premise, it has been reported that in mice harboring mutations inactivating the NPR-C, 'normal body fat deposits are absent' [28]. Thus, adipocyte-derived Ostn could modulate the response of fat to insulin and ANP signaling under pathophysiological conditions. It has yet to be determined if adipocyte Ostn is regulated by nutritional status or insulin, as it is in muscle. The presence of Ostn in both fat and muscle presents the molecule as a potential mediator of muscle effects on fat regulation. Recently, a constitutively active form of AKT1 in fast twitch muscle fibers, those that express high Ostn levels, has been shown in mice to induce muscle cell hypertrophy and to reduce fat deposits [29]. It was hypothesized that muscle-derived circulating factors could act distally on lipid metabolism. Ostn could be one such factor since AKT1 is predicted to be an upstream activator of its gene expression. Such a possibility of Ostn acting as a "myokine" with actions on distal tissues such as liver and fat to regulate whole body homeostasis is a very interesting possibility (Fig. 1B), and presents an intriguing correlate with the recent discovery that osteocalcin is a crucial bone-derived hormone involved in the regulation of whole body energy metabolism [30]. Development of assays to measure circulating Ostn would shed light on this issue.

Future perspectives

Although we have elucidated the basic molecular mechanism of Ostn action, several aspects of its biochemistry still remain unclear. For instance, what convertase/protease cleaves Ostn and what is the ultimate outcome – activation and/or degradation? Furin, a serine protease that also activates CNP, is one such likely candidate. Is the full-length Ostn and/or the ensuing peptides functional and do they exist *in vivo?* Is the processing the same in different tissues? Is there a circulating form of Ostn and is it functional? Are Ostn's effects in muscle and fat mediated through the natriuretic system?

Ostn potentially presents a new mechanism linking a number of mesenchymal tissues with the natriuretic system, possibly further integrating the cross-talk and inter-dependency of these different tissues. Targeted disruption of the Ostn gene will further help decipher its effects in these tissues.

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