MINI REVIEW

Title:

The Bioactive Acidic Serine- and Aspartate-Rich Motif Peptide

Authors:

Tomoko Minamizaki and Yuji Yoshiko*

Department of Calcified Tissue Biology, Hiroshima University Institute of Biomedical & Health Sciences, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan

* Address for correspondence:

Yuji Yoshiko, Ph.D.

Department of Calcified Tissue Biology, Hiroshima University Institute of Biomedical & Health Sciences, 1-2-3, Kasumi, Minami-ku, Hiroshima 734-8553, Japan; Tel/Fax: +81 82 257 5620, +81 82 257 5621; E-mail: yyuji@hiroshima-u.ac.jp

Abstract:

The organic component of the bone matrix comprises 40% dry weight of bone. The organic component is mostly composed of type I collagen and small amounts of non-collagenous proteins (NCPs) (10-15% of the total bone protein content). The small integrin-binding ligand N-linked glycoprotein (SIBLING) family, a NCP, is considered to play a key role in bone mineralization. SIBLING family of proteins share common structural features and includes the arginine-glycine-aspartic acid (RGD) motif and acidic serine- and aspartic acid-rich motif (ASARM). Clinical manifestations of gene mutations and/or genetically modified mice indicate that SIBLINGs play diverse roles in bone and extraskeletal tissues. ASARM peptides might not be primary responsible for the functional diversity of SIBLINGs, but this motif is suggested to be a key domain of SIBLINGs. However, the exact function of ASARM peptides is poorly understood. In this article, we discuss the considerable progress made in understanding the role of ASARM as a bioactive peptide.

Keywords:

Acidic serine- and aspartic acid-rich motif (ASARM), Bone mineralization, Matrix extracellular phosphoglycoprotein (MEPE), Small integrin-binding ligand N-linked glycoprotein (SIBLING).

1. INTRODUCTION

The extracellular bone matrix, unlike most connective tissues, is physiologically mineralized, and constantly reconstructed (bone turnover) throughout life. Osteoblasts, the bone-forming cells, are derived from mesenchymal stem cells, while the bone-resorbing osteoclasts originate from hematopoietic stem cells. Osteocytes are terminally differentiated osteoblasts embedded in mineralized bone and make up over 90% of bone cells that greatly outnumber osteoblasts and osteoclasts. Bone homeostasis is maintained by the balance between osteoblastic bone formation and osteoclastic bone resorption, and is closely correlated with the osteocytic cell-cell networks [1]. Therefore, any dysfunction in these cells alters bone mass and architecture. Bone formation proceeds via intramembranous and endochondral ossification. Intramembranous bone formation takes place when osteo-chondrogenic progenitor cells differentiate directly into osteoblasts, while endochondral ossification happens when the progenitor cells differentiate into chondrocytes to form a cartilage template that is subsequently replaced by bone. In both cases, osteoblasts actively participate in bone formation processes, such as extracellular matrix (ECM) deposition, matrix maturation and mineralization that is under the control of a multitude of local and systemic factors [2].

The ECM in bone is composed of minerals, collagen fibers, water, noncollagenous proteins (NCPs), and lipids. Hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] is a naturally occurring form of mineral, in which the elements are substituted with carbonate, magnesium and others, depending on the diet and environment. Type I collagen is the most abundant component of bone matrix proteins and serves as a basic building block of the matrix fiber network. NCPs contain serum-derived proteins, proteoglycans, glycosylated proteins, γ-carboxyglutamic acid-containing proteins, growth factors, and small integrin-binding ligand, N-glycosylated proteins (SIBLINGs) [3]. Human diseases and/or animal models show that along with the most abundant form of NCP that is osteonectin (approximately 2% of total protein of developing bone) [4], small amounts of NCPs, *e.g.*, SIBLINGs [5-8] also contribute to the structural and biochemical

properties of the bone.

The SIBLING family includes dentin matrix protein 1 (DMP1), dentin sialophosphoprotein (DSPP), integrin binding sialoprotein (IBSP, which encodes bone sialoprotein, BSP), matrix extracellular phosphoglycoprotein (MEPE), and secreted phosphoprotein 1 (SPP1, which encodes osteopontin, OPN). The encoding genes for these proteins are all located on human chromosome 4q21 and mouse chromosome 5q with similar exon structures, and these members are probably a result of early gene duplication and divergence [9]. The SIBLINGs are poorly conserved at the amino acid sequence level, while all of them are known as ECM proteins and defined by the conserved motifs. The RGD (Arg-Gly-Asp) motif and post-translational modification motifs that include casein kinase phosphorylation, glycosylation, and proteolysis, are reflected in the functional significance of these proteins. SIBLINGs rich in hydrophilic amino acids are likely to be extended and flexible structures in solution, and link extracellular protein-protein interactions with the intracellular cytoskeleton. Thus, it participates in cell migration, proliferation, differentiation and so on. Recently, it has been proposed that a new functional domain, acidic serine- and aspartate-rich motif (ASARM) acts as an inhibitor of matrix mineralization. SIBLING family members have been extensively reviewed before (see for example, [9, 10]), while roles of ASARM are controversial. Growing evidence indicates that ASARM peptide fragments may be biologically active and more than just a waste product of bone formation and resorption. In this article, we will focus on our current understanding of ASARM and discuss a possible role of this peptide in bone.

2. DIVERSE FUNCTIONS OF SIBLING PROTEINS: SPP1 (OPN) *vs.* MEPE

OPN is also known as bone sialoprotein I and early T-lymphocyte activation 1. The gene encoding this protein was first isolated in 1986 from the cDNA library of a rat osteosarcoma cell line (ROS17/2.8) [11]. In normal conditions, *Opn* is constitutively expressed in bone and kidney, and also detected in extraskeletal tissues/cells such as

epithelial cells of the gastrointestinal tract, gallbladder, pancreas, reproductive tract, lung, breast, salivary glands, sweat glands, ganglion cells [12], activated $CD4^+$ T cells and macrophages [13]. OPN is a 33-kDa nascent protein (314 amino acids in human and 297 amino acids in mouse) and goes through posttranslational modifications by glycosylation and phosphorylation, thereby increasing its molecular weight to 44-kD and over. Approximately 30 phosphorylation sites exist in this polypeptide, while their usage varies among tissues/cells. Full-length OPN can be cleaved by thrombin [14] and matrix metallopeptidase (MMP)-3 and MMP-7 [15]. It is also known that transglutaminase catalyzes formation of cross-linking of OPN to collagen calcium-dependently [16]. Transcription factor nuclear factor κB signaling is involved in OPN activities (see for example,[17]). Thus, OPN exists both as an immobilized ECM molecule and as a cytokine in body fluids [18], and is involved in various biological activities including skeletal mineralization [8], bone remodeling [19], cell migration, and activation of immunocompetent cells [20, 21]. The above-mentioned OPN pathways are also supposed to be closely correlated with rheumatoid arthritis [22], allergic airway disease [23], and cancer cell progression (including transformation, invasion and metastasis) [9].

In comparison to OPN, there is limited information on MEPE. *MEPE* was identified from a cDNA library of hemangiopericytoma, one of mesenchymal tumors responsible for oncogenic hypophosphatemic osteomalacia (OHO) [24]. OHO sharers pathologic conditions with inherited hypophosphatemic rickets/osteomalacia (*e.g.*, X-linked hypophosphatemia, XLH) [25]. As a common etiology in these diseases, circulating phosphaturic factor “phosphatonin” has been explored as a substrate for phosphate-regulating neutral endopeptidase, X-linked (PHEX) in which loss-of-function mutations cause XLH [26]. *Mepe* is expressed in human [27] and mouse bone [25] and dentin [28], especially in fully differentiated mouse osteoblasts. The human *MEPE* gene encodes 525-amino acid protein that shares only 50% sequence identity with mouse counterpart consisting of 433 amino acids [25]. However, conserved motifs, such as RGD cell attachment sequence, a SGDG glycosaminoglycan attachment motif, N-glycosylation sites, and multiple phosphorylation sites exist across these species [24,

25]. It is postulated that alternative splicing of the human *MEPE* gene results in multiple transcript variants. MEPE was initially thought to be a candidate for phosphatonin, a possible substrate for PHEX, while its biological properties are controversial.

MEPE deficiency does not impinge serum phosphate levels in mice, and rather increases trabecular bone mass [5]. Overexpression of *Mepe* in mice driven by Col1a1 2.3-kb promoter decreases bone volume and causes hyperphosphatemia via the upregulation of the renal sodium-dependent phosphate cotransporter *Npt2a* [29]. It is to be noted that in this transgenic model, *Mepe* is overexpressed not only in bone but also in kidney [29, 30]. *Mepe* is highly expressed in the bones of a mouse XLH model (*Hyp*) [25, 31], while PHEX fails to hydrolyze MEPE in a cell-free system [31]. Backcrossing *Mepe*-deficient mice onto *Hyp* mice fails to correct hypophosphatemia, demonstrating that MEPE is not phosphatonin [32]. In contrast, insect-expressed recombinant human MEPE (i-rhMEPE) is capable of causing hypophosphatemia and/or hyperphospaturia in mice [33] and rats [34], possibly by promoting intestinal phosphate uptake with the increased protein levels of NPT2a [35]. MEPE is in circulation in healthy adult and elderly humans, whose levels are correlated with those of serum phosphate, parathyroid hormone (PTH), and bone mineral density [36]. Serum MEPE levels appear to be mostly within concentrations less than 600 ng/ml in 114 normal human subjects [36], therefore, the effects of MEPE on phosphate homeostasis may differ between normal and pathological conditions. Also, the action of MEPE on osteoclasts is inconclusive, as no association is found in bone marrow cell cultures (inhibitory effect) [37] versus *Mepe*-null mice (no effect) [5].

ASARMs

ASARMs rich in aspartate and glutamate amino acid residues acquire a net negative charge, resulting in their ability to bind calcium ions and calcium-containing inorganic mineral crystals in bone, cartilage and teeth. Therefore, SIBLINGs/ASARMs may contribute to the formation of calcium phosphate/hydroxyapatite crystals positively or negatively. As described before, MEPE-ASARM exhibits less than 80 % of sequence similarity to other ASARMs [24]. Figure 1 shows the sequence alignments and positions between MEPE-ASARM and other SIBLING family members (DSPP, DMP1,

BSP, and OPN), based on the 50% and more sequence identity in 14-19-residue overlaps. DSPP is mostly cleaved into two major proteins, dentin sialoprotein (DSP) and dentin phosphoprotein (DPP) [28, 38], and the latter contains a long repeat of ASARM and RGD motifs. A deletion of *Dpp* results in roughly similar *Dspp*-null mouse phenotypes exhibiting defects in dentin mineralization [39]. Unlike DSP, DPP appears not to be processed by matrix metalloproteinases [40], and thus the involvement of DPP-ASARM in mechanisms underlying *Dpp*-null dentin hypomineralization remains unclear. Observations of mice lacking *Opn* [8] but not *Bsp* [7] and *Dmp1* [41] indicate the role of ASARM as an inhibitor of mineralization. Multiple active sites including ASARM are distributed differentially in the complete sequence of SIBLING family members, suggesting that not all ASARMs in these proteins may be equally active. In this respect, only OPN- [30] and MEPE-ASARMs (see below) have been examined so far. Phosphorylation of serine residues is also involved in its calcium binding activity, and rat bone OPN contains an average of eleven phosphorylation sites out of 29 potential sites [42]. Based on predicted phosphorylated residues in human OPN by NetPhosK 1.0 Server (http://www.cbs.dtu.dk/services/NetPhosK/), a maximum of three serine residues in human OPN-ASARM ($OPN_{3(p)S}$-ASARM) may be phosphorylated (Figure 2). A synthetic peptide corresponding to $OPN_{3(p)S}$-ASARM (syn$OPN_{3(p)S}$-ASARM) at the concentrations of 10 μM or more inhibits mineralized matrix formation in the mouse osteoblast cell line MC3T3-E1, which is abrogated by addition of mammalian-expressed soluble recombinant human PHEX (truncated PHEX lacking the membrane domain, rhsPHX). Phosphoserine in OPN-ASARM is necessary for this process and binding to synthetic hydroxyapatite. synOPN-ASARM with or without phosphoserine does not show significant effects on cell proliferation and differentiation in this model.

3. MEPE-ASARM

As shown in Figure 2, MEPE-ASARM is highly conserved among mammalian species and exhibits a unique feature being location at the C-terminal end.

Phosphorylation of serine 514 (S514), S518, S520, and S522 in human MEPE-ASARM amino acid sequence is postulated to be in common with other species. MEPE appears to be cleaved at regions close to ASARM, in which cathepsin B produced by osteoblasts can act and result in release of an ASARM peptide fragment (with and/or without phosphorylation) into the skeletal microenvironment [31], and circulation [43]. Circulating levels of MEPE-ASARM and its accumulation in bone [43, 44] and dentin [45] are significantly higher in *Hyp* mice and patients with XLH, while this peptide is also located in the osteoid and young osteocytes of normal mouse bone [44]. Injections of synthetic hMEPE peptide fragment (AC-100, 242-264, 200 μg/kg/day and less), containing RGD and glycosaminoglycan-attachment motifs, over the calvariae for 5 days increase bone formation in newborn mice [37]. Likewise, AC-100 (3 μg/ml) and *E.Coli*-derived ASARM-truncated rhMEPE has anabolic effects on rat calvaria cell cultures by promoting osteoprogenitor cell adhesion [46]. Thus, it is clear that MEPE-ASARM does not participate in MEPE-dependent increase in bone formation. Intraperitoneal bolus injection of insect-expressed i-rhMEPE (400 μg/kg/30h) causes hypophosphatemia in mice possibly due to its inhibitory effect on renal phosphate reabsorption [33], this is in agreement with the results of transgenic mice overexpressing *Mepe* [29]. Daily subcutaneous injections of synMEPE-ASARM with phosphorylated S518, S520 and S522 (synMEPE$_{3(p)S}$-ASARM, 5mg/kg/day) increase osteoid thickness and hypomineralization in calvariae and long bones [47]. Further, a continuous infusion of synMEPE$_{3(p)S}$-ASARM (2mg/kg/day via osmotic pump) into 20-week-old mice for 4 weeks decreases serum phosphate and renal *Npt2a* mRNA levels. However, it increases serum levels of fibroblast growth factor 23 (FGF23), a phosphaturic factor, and decreases levels of osteocalcin and OPN [48]. Implantation of Affi-Gel agarose beads soaked in synMEPE$_{3(p)S}$-ASARM (2 μg/μl) in dental pulps delays wound healing in a rat injured dentin/pulp model [45].

In vitro studies on rhMEPE and synMEPE-ASARM are complicated. Not only i-rhMEPE (≥100 ng/ml) but also synMEPE-ASARM (human sequence, ≥60 ng/ml) without any phosphorylation inhibits mineralized matrix formation in the mouse osteoblast cell line 2T3 in the presence of bone morphogenetic protein 2 [33]. However,

nonphosphorylated synMEPE-ASARM fails to decrease mineralized matrix formation in mouse bone marrow stromal cell cultures [49]. Again, synMEPE$_{3(p)S}$-ASARM (≥1 μM) but not synMEPE-ASARM inhibit mineralized matrix formation in a MC3T3-E1 mouse osteoblast cell line [44].

4. CONSIDERATIONS FOR MECHANISM(S) OF MEPE-ASARM ACTIONS

The precise mechanism(s) of MEPE-ASARM actions as above remain controversial. Since MEPE is originally identified in tumors involved in OHO and is a candidate for "phophatonin" [24], it is not surprising that MEPE decreases renal phosphate reabsorption [29, 33]. Under pathogenic conditions, excess amounts of MEPE can cause phosphaturia and hypophosphatemia, resulting in skeletal anomalies. However, it is notable that synMEPE$_{3(p)S}$-ASARM can mimic these effects [47, 48], raising question about how synMEPE$_{3(p)S}$-ASARM inhibits renal phosphate reabsorption and bone formation. The former issue remains poorly understood. Like MEPE, is synMEPE$_{3(p)S}$-ASARM targeted to *Npt2a*? What are the signaling pathway(s) in MEPE- and MEPE-ASARM-target cells in kidney? Of the latter issue, there are inconsistent findings across studies; synMEPE$_{3(p)S}$-ASARM inhibits mineralized matrix formation with no effect on cell proliferation and differentiation in MC3T3-E1 cells [44]. However, the synthetic peptide inhibits the expression of principal osteoblast/osteocyte-specific genes, such as *Runx2*, *Phex*, and *Dmp1*, while it increases *Mepe* and osteoprotegerin mRNA levels in mouse bone marrow stromal cells [49]. It is unclear whether the difference between the two is dependent on their lineage and/or developmental stage backgrounds or undefined reasons. If it does, how MEPE-ASARM regulates the expression of osteoblast/osteocyte-specific genes is notable. Whether phosphoserine in ASARM is necessary for its actions appears to be settled by recent reports including OPN-ASARM [30], but further studies are needed to evaluate the role(s) of nonphosphorylated ASARM (see below). The majority of the actions of synMEPE$_{3(p)S}$-ASARM on bone formation may be facilitated during mineralization processes by inhibiting hydroxyapatite crystal growth [44]. This event totally coincides

with the chemical features (negatively charged amino acids) of ASARM as above.

As described, MEPE has been postulated to be associated with PHEX [24]. Baculovirus-expressed rhPHEX fails to hydrolyze *E.Coli*-derived rhMEPE but protects MEPE protein from cathepsin B proteolysis in a cell-free system [31]. This finding is consistent with the increased levels of MEPE-ASARM in bone and circulating MEPE-ASARM in *Hyp* mice [43, 44]. rhsPHEX interacts not only with i-rhMEPE but also with synMEPE-ASARM with and without phosphoserine, as measured by surface plasmon resonance analysis [47]. Both i-rhMEPE (Ki=2.1 nM) and synMEPE$_{3(p)S}$-ASARM (Ki=128.7 nM) inhibit rhsPHEX enzyme activity in a cell-free system and increase the expression of FGF23 in mouse bone marrow cell cultures [50]. On the contrary, rhsPHEX almost completely reverses the inhibitory effect of synOPN$_{3(p)S}$-ASARM on mineralized matrix formation in MC3T3-E1cells, and cleaves both human and mouse ASARM peptides (with and without phosphoserine) at the amide linkages between serine and glutamate or between serine and aspartate in a cell-free system [44]. A small synthetic PHEX peptide capable of binding to synMEPE$_{3(p)S}$-ASARM also ameliorates the inhibitory effect of synOPN$_{3(p)S}$-ASARM in mouse bone marrow cells [49]. Because i-rhPHEX fails to hydrolyze i-rhMEPE in a cell-free system [31], ASARM, a cleavage product of MEPE by cathepsin B [31] may be further processed by PHEX. Thus, MEPE-ASARM in the presence and absence of phosphoserine may interact with PHEX, while phosphorylation of certain serine residues in ASARM (see above) may modulate its effects on bone formation. It may be needed to know more about nonphosphorylated versus phosphorylated MEPE-ASARM than phosphorylated MEPE-ASARM alone *in vivo*. Interestingly, MEME-ASARM is also involved in osteoclastogenesis [49], chondrogenesis [51] and odontogenesis [45].

CONCLUSIONS

Little is known about whether ASARM peptide fragment, a consensus sequence motif in SIBLING family members acts as a bioactive peptide. As summarized in Figure 3, MEPE-ASARM may inhibit bone mineralization primarily by binding to hydroxyapatite crystals through its negatively charged properties, while the peptide may interact with PHEX enzyme involved in phosphate metabolism. It is possible that

ASARM is also targets progenitor cells, such as osteogenic cells and dental pulp cells and regulates their development via an undefined mechanism. MEPE is processed, then nonphosphorylated and phosphorylated ASARMs are accumulated in bone, and released into circulation, suggesting that additional role(s) of ASARM. Further research is necessary to establish the precise role(s) of ASARM *in vivo*.

FIGURE LEGENDS

Figure 1. Amino acid sequence identity (%) between human MEPE-ASARM and other human SIBLINGs. Numbers show positions in the indicated amino acid sequences through the NCBI database. The asterisks denote identical amino acid residues.

Figure 2. Predicted phosphorylation sites of human OPN and MEPE by casein kinase. Underlines indicate positions of ASARMs. Mouse, rat (OPN and MEPE), cattle and tarsier (MEPE) ASARMs are also listed. S (green) is phosphorylated by casein kinase I; S/T (red) are phosphorylated casein kinase II or I. Phosphoserines 106, 112 and 115 of human OPN and 514, 518, 520 and 522 of human MEPE are conserved among species.

Figure 3. Possible actions of ASARM on bone. MEPE produced by osteoblasts can bind to cell membrane protein including integrin and extracellular matrix (ECM) and influence multiple cell activities. Osteoblast-derived cathepsin B cleaves MEPE, with the resultant release of ASARM peptide fragment. Not only MEPE but also ASARM interacts with PHEX, which may affect their own activities on each other. ASARMs with and without phosphoserine are degraded by PHEX, while the reduced activity of PHEX increases levels of ASARM in bone as well as in circulation. The accumulation of phosphorylated ASARM in bone that binds to hydroxyapatite and inhibits its crystal growth. Thus phosphorylated ASARM acts as an inhibitor of bone mineralization. Phosphorylated ASARM also regulates osteoblast-specific genes via an undefined mechanism, resulting in alteration of bone metabolism. As MEPE is, circulating phosphorylated ASARM is delivered to extraskeletal organs and somehow affects target cells.

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Figure 1. Sequence Identity (%) between MEPE-ASARM and other SIBLINGs.

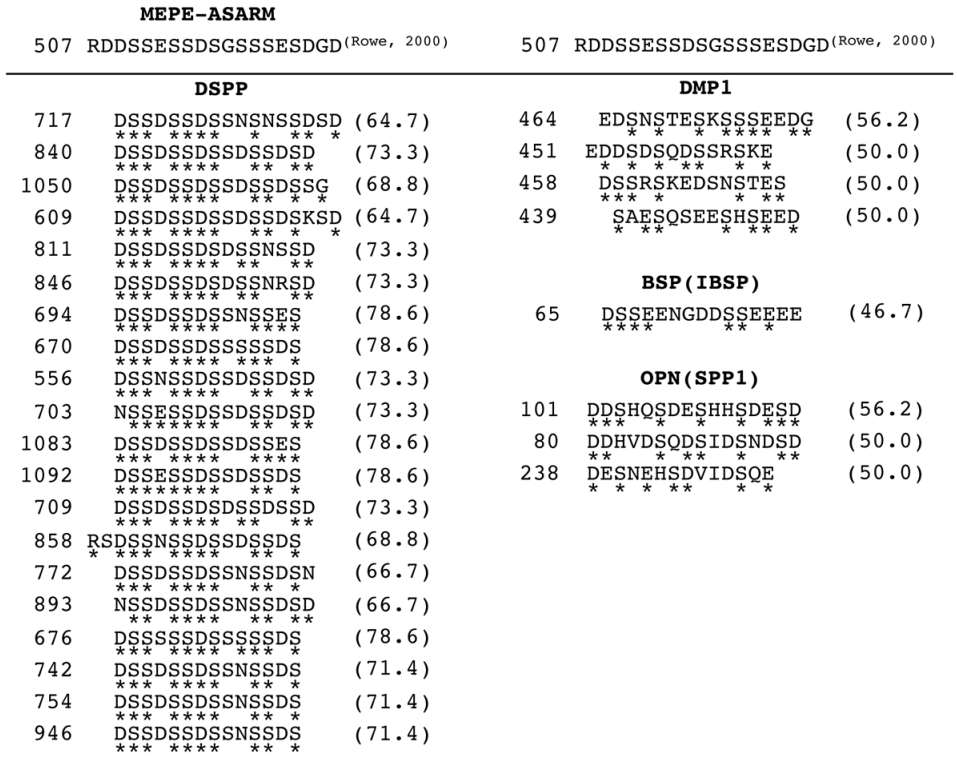


Figure 2. Predicted phosphorylation sites of OPN and MEPE by casein kinase

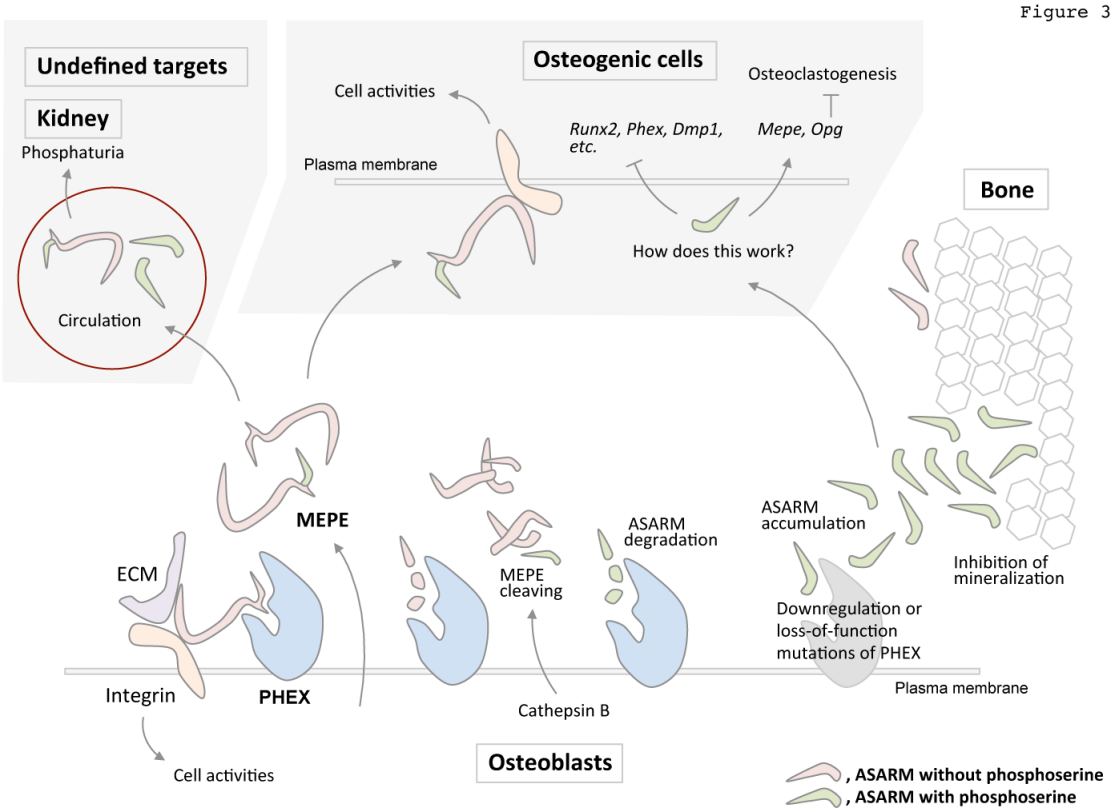
Human OPN

```
1        10        20        30        40        50        60        70        80
MRIAVICFCLLGITCAIPVKQADSGSSEEKQLYNKYPDAVATWLNPDPSQKQNLLAPQTLPSKSNESHDHMDDMDDEDDD
81       90        100       110       120       130       140       150       160
DHVDSQDSIDSNDSDDVDDTDDSHQSDESHHSDESDELVTDFPTDLPATEVFTPVVPTVDTYDGRGDSVVYGLRSKSKKF
                     VDSDESDESHHSDESDET 121 (Mouse)
                     VNSDESDESHHSDESDES 121 (Rat)
161      170       180       190       200       210       220       230       240
RRPDIQYPDATDEDITSHMESEELNGAYKAIPVAQDLNAPSDWDSRGKDSYETSQLDDQSAETHSHKQSRLYKRKANDES
241      250       260       270       280       290       300
NEHSDVIDSQELSKVSREFHSHEFHSHEDMLVVDPKSKEEDKHLKFRISHELDSASSEVN
```

Human MEPE

```
1        10        20        30        40        50        60        70        80
MRVFCVGLLLFSVTWAAPTFQPQTEKTKQSCVEEQRQEEKNKDNIGFHHLGKRINQELSSKENIVQERKKDLSLSEASEN
81       90        100       110       120       130       140       150       160
KGSSKSQNYFTNRQRLNKEYSISNKENTHNGLRMSIYPKSTGNKGFEDGDDAISKLHDQEEYGAALIRNNMQHIMGPVTA
161      170       180       190       200       210       220       230       240
IKLLGEENKENTPRNVLNIIPASMNYAKAHSKDKKKPQRDSQAQKSPVKSKSTHRIQHNIDYLKHLSKVKKIPSDFEGSG
241      250       260       270       280       290       300       310       320
YTDLQERGDNDISPFSGDGQPFKDIPGKGEATGPDLEGKDIQTGFAGPSEAESTHLDTKKPGYNEIPEREENGGNTIGTR
321      330       340       350       360       370       380       390       400
DETAKEADAVDVSLVEGSNDIMGSTNFKELPGREGNRVDAGSQNAHQGKVEFHYPPAPSKEKRKEGSSDAAESTNYNEIP
401      410       420       430       440       450       460       470       480
KNGKGSTRKGVDHSNRNQATLNEKQRFPSKGKSQGLPIPSRGLDNEIKNEMDSFNGPSHENIITHGRKYHYVPHRQNNST
481      490       500       510       520
RNKGMPQGKGSWGRQPHSNRRFSSRRRDDSSESSDSGSSSESDGD 525
                           RDSSESSSSGSSSESHGD 433 (Mouse)
                           RDSSESSSSGSSSESSGD 435 (Rat)
                           HDSSESSDSGSSSESDGD 554 (Cattle)
                           DDSSESSDSSSSSESNGD 560 (Tarsier)
```

Figure 3.


Undefined targets
Kidney
Phosphaturia
Circulation
Osteogenic cells
Cell activities
Plasma membrane
Runx2, Phex, Dmp1, etc.
Osteoclastogenesis
Mepe, Opg
How does this work?
Bone
MEPE
ECM
Integrin
PHEX
Cell activities
MEPE cleaving
ASARM degradation
Cathepsin B
Osteoblasts
ASARM accumulation
Downregulation or loss-of-function mutations of PHEX
Inhibition of mineralization
Plasma membrane
, ASARM without phosphoserine
, ASARM with phosphoserine